Mitochondrial Glutathione Transport: Implications for Bcl-2 and Neuronal Survival

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Mitochondrial Glutathione Transport: Implications for Bcl-2 and Neuronal Survival

Abstract

Oxidative stress is a contributing factor to many neurodegenerative diseases. In particular, mitochondria are a key source of oxidative stress due to electron leakage at the level of the electron transport chain. To combat the endogenous production of reactive oxygen and reactive nitrogen species mitochondria are equipped with several redox-cycling systems, such as glutathione (GSH). Mitochondrial GSH has been shown to be a critical reservoir of this antioxidant, where selective depletion of mitochondrial GSH can induce apoptosis in several systems. Many studies have intricately linked Bcl-2 to cellular GSH status and it has been previously shown that Bcl-2 is a GSH binding protein. Therefore, we hypothesize that Bcl-2 may play an important role in regulating mitochondrial GSH transport.

Here, we show that inhibition of Bcl-2 in primary cerebellar granule neurons (CGNs) induced apoptosis, mitochondrial oxidative stress, selective depletion of the mitochondrial GSH pool, and led to inhibition of mitochondrial GSH transport. Furthermore, we found that Bcl-2 is an interacting partner with a previously identified mitochondrial GSH transporter, the 2-oxoglutarate carrier (OGC), and this interaction is modulated by GSH. To further support a role for Bcl-2 in regulating mitochondrial GSH transport, we show that Bcl-2 requires OGC for protection against apoptosis induced by oxidative stress and to increase the mitochondrial GSH pool. These data suggest that Bcl-2 plays a key role in regulating mitochondrial GSH transport and that the enhancement of the interaction between Bcl-2 and OGC by GSH may increase mitochondrial GSH transport.

The mechanisms of mitochondrial GSH transport thus far have only been extensively studied in liver and kidney, where the OGC and the dicarboxylate (DIC) carrier have been identified as inner membrane mitochondrial GSH transporters. Most studies examining mitochondrial GSH transport mechanisms in brain have not allowed for the distinction between neuronal and glial cell mitochondrial GSH transport mechanisms. Therefore, we employed primary cerebellar astrocytes and primary CGNs as a system to study mitochondrial GSH transport mechanisms and differences between neuronal and glial cells. It was found that cerebellar astrocytes use both the OGC and DIC to transport GSH into mitochondria, while CGNs preferentially use the DIC. In addition, discrete inhibition of one mitochondrial GSH transporter (DIC) using chemical inhibition and genetic knockdown, in CGNs, led to increased susceptibility to both oxidative and nitrosative stress. Overall, this suggests that inhibition of a single mitochondrial GSH transporter is sufficient to predispose neurons to oxidative stress, such as the conditions observed in neurodegenerative diseases.

Finally, the consequences of overexpressing a mitochondrial GSH transporter were examined. Stable NSC34 motor neuron-like cell lines were produced which overexpress OGC. The stable OGC cell lines had significantly increased mitochondrial GSH levels and were markedly resistant to both oxidative and nitrosative stress. More importantly, the stable OGC cell lines up-regulated Bcl-2 expression (a protein we have shown previously to interact with OGC) and this phenomenon was due to increased mitochondrial GSH levels. The up-regulation of Bcl-2 was required to sustain increased mitochondrial GSH levels and resistance to oxidative stress. Overall, this study implicates the importance of not only the mitochondrial GSH pool, but mitochondrial GSH transport for neuronal survival. These findings suggest that modulation of mitochondrial GSH transport could be a novel therapeutic approach for neurodegenerative diseases.

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Mitochondrial Glutathione Transport: Implications for Bcl-2 and Neuronal Survival

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CHAPTER ONE: INTRODUCTION

Mitochondrial dysfunction, oxidative stress, and subsequent apoptosis are major unifying themes in the pathology of neurodegenerative diseases. Particularly, oxidative stress at the level of the mitochondria induces mitochondrial dysfunction and downstream apoptosis. Glutathione (GSH) is a key endogenous antioxidant compartmentalized into mitochondria, where it functions to combat reactive oxygen species (ROS) which are produced by the electron transport chain (ETC) as a byproduct of aerobic energy production. The mitochondrial GSH pool has been shown to be critical for cell survival in numerous systems. However, the precise role that mitochondrial GSH transport plays in the maintenance of cell viability remains to be understood. The mechanism of GSH compartmentalization into mitochondria has been extensively studied in kidney and liver, while limited studies have been completed regarding the same mechanisms within the central nervous system (CNS). Furthermore there is a lack of focus on the regulation of mitochondrial GSH transport. Prevalent evidence suggests a role for Bcl-2 in GSH trafficking and metabolism, and it has been previously shown that Bcl-2 is a GSH binding protein (Voehringer et al., 1998; Zimmermann et al., 2007). A major focus of this thesis is to examine the possible role of Bcl-2 in the regulation of mitochondrial GSH transport while also elucidating the importance of mitochondrial GSH transport in neuronal survival.
1.1 Overview of Apoptotic Pathways and their Involvement in Neurodegenerative Diseases

Apoptosis, or programmed cell death, plays important physiological roles in the development and maintenance of an organism; however, aberrant signaling to or from apoptotic pathways contributes to the pathogenesis of diverse diseases. An overabundance of apoptosis for example, leads to neurodegenerative diseases while a lack of apoptosis is observed in many types of cancer. Currently, it is understood that two distinct apoptotic pathways exist, the extrinsic and intrinsic apoptotic pathways; both are discussed below and reviewed in Figure 1.1.

1.1.1 The Extrinsic Apoptotic Pathway

The extrinsic apoptotic pathway initiates with an extracellular signal, such as that from cytotoxic T cells of the immune system. This extracellular signal leads to the recruitment and dimerization of death receptors (i.e. TNF or Fas receptors) which are specific for the extracellular signal received (Elmore, 2007; Ow et al., 2008). The oligomerization of the death receptors initiates the intracellular formation of the death inducing signaling complex (DISC) which, subsequently recruits procaspase-8 and activates it through a cleavage event (Chang et al., 2003; Donepudi et al., 2003; Cordeiro and Bidere, 2013). Other procaspases are cleaved downstream and activated by caspase-8, including caspase-3 and -9. In addition, caspase-8 cleaves the BH3-only protein, Bid, producing its activated form, truncated-Bid (t-Bid) (Li et al., 1998). This mechanism is considered a convergence point for the extrinsic and intrinsic apoptotic pathways, as t-Bid also translocates to the outer mitochondrial membrane, initiating the intrinsic or mitochondrial apoptotic cascade, as discussed in detail below.
1.1.2 The Intrinsic Apoptotic Pathway

Intrinsic apoptosis, or the mitochondrial apoptotic pathway, is initiated by an intracellular signal such as DNA damage, accumulation of misfolded proteins, or oxidative stress. The exact mechanisms of this pathway are highly debated; here some of the major hypotheses will be reviewed. The Bcl-2 family of proteins, both pro-survival (Bcl-2, Bcl-XL, Bcl-w) and pro-apoptotic, (Bax and Bak multi-domain proteins; and BH3-only proteins such as Bim, Bad, Hrk) have been shown to regulate the mitochondrial apoptotic pathway (Figure 1.2; Burlacu, 2003). This pathway involves the activation of BH3-only proteins, such as the dephosphorylation of Bad, which causes its release from 14-3-3 plasma membrane anchoring proteins, thus allowing its translocation to mitochondria (Masters et al., 2001; Yang et al., 2008). There are two main hypotheses regarding the mechanism by which BH3-only proteins promote apoptosis at mitochondria. BH3-only proteins are thought to either inhibit pro-survival Bcl-2 family proteins (such as Bcl-2 and Bcl-XL) thus “sensitizing” mitochondria to the effects of Bax and Bak (indirect model); or to directly activate Bax and Bak (direct model) (Cheng et al., 2001; Vela et al., 2013). However, evidence has been provided which unifies both hypotheses suggesting that certain BH3-only proteins may act as suppressors of pro-survival proteins while others may act as direct activators of Bax and Bak (Kuwana et al., 2005; Kim et al., 2006). Following the activation of BH3-only proteins, the mitochondrial outer membrane becomes permeabilized, leading to the release of pro-apoptotic proteins such as cytochrome c and the apoptosis inducing factor (AIF) (Green and Reed, 1998; Martinour and Green, 2001). Two main hypotheses dominate the mechanism by which mitochondrial outer membrane permeabilization (MOMP) occurs. First, is direct pore
formation by the pro-apoptotic proteins Bax and Bak, where previous studies have shown
the ability of Bax to form pores in membranes and induce MOMP in isolated
mitochondria (Antonsson et al., 2000; von Ahsen et al., 2000; Desagheer and Martinou,
2000; Suzuki et al., 2000). Second, in the mitochondrial permeability transition pore
(mPTP) model, matrix swelling is caused by the loss of mitochondrial membrane
potential and inward flow of ions, such as Ca\(^{2+}\). The mPTP is thought to be responsible
for the loss of mitochondrial membrane potential and is hypothesized to consist of
voltage dependent anion channel (VDAC), adenine nucleotide translocase (ANT), and
cyclophilin D (Crompton et al., 1998; Crompton et al., 1999). Finally, these two
hypotheses are linked by studies showing that Bax and Bak interact with components of
the proposed mPTP, VDAC and ANT, thus suggesting there is cooperation between pro-
apoptotic Bcl-2 family proteins and the mPTP in the permeabilization of the outer
mitochondrial membrane and release of mitochondrally sequestered pro-apoptotic
proteins (Marzo, 1998; Shimizu et al., 1999; Tsujimoto and Shimizu, 2002). In addition
to the two hypotheses regarding MOMP mechanisms, the release of cytochrome c from
cardioliopin (the lipid which tethers cytochrome c to the inner mitochondrial membrane)
can also occur through several mechanisms. First, is the direct oxidation of cardioliopin by
enhanced ROS production or the cardioliopin-cytochrome c complex (Ott et al., 2002;
Orrenius and Zhivotovsky, 2005). Second, increased mitochondrial Ca\(^{2+}\) can weaken the
electrostatic interaction between cardioliopin and cytochrome c and lastly, hydrolysis of
cardioliopin by phospholipase A2 can lead to the release of cytochrome c from
mitochondria (Sedlack and Robinson, 1999; Ott et al., 2007). Overall, cytochrome c
discharge results in electron transport deficits, further production of ROS, and even
greater release of cytochrome c into the cytosol (Orrenius et al., 2007). Once released, cytochrome c associates with the apoptotic protease-activating factor (Apaf-1), which induces the cleavage and activation of pro-caspase-9, forming the apoptosome complex, and downstream activation of other caspases and caspase activated DNAses (CADs) (Zou et al., 1999; Adrain and Martin, 2001; Jiang and Wang, 2000; Kim et al., 2005; Riedl and Salvesen, 2007). The apoptotic pathway culminates in membrane blebbing, degradation of intracellular proteins and DNA, presentation of extracellular apoptotic markers (phosphatidylserine) and phagocytosis of the apoptotic cell, thus preventing damage to other neighboring cells and creating a “silent” death lacking significant inflammation (Fadok et al., 1992; Lauber et al., 2004).
**Figure 1.1 Overview of Apoptotic Pathways** The extrinsic and intrinsic pathways are depicted, showing the binding of the death receptor (Fas) by the death ligand (FasL), intracellular signaling through the DISC complex, activation of caspases (Pro-Cas to Cas), inhibition of inhibitor of caspase activated DNAses (ICAD) and thus activation of caspase activated DNAses (CADs), leading to the degradation of DNA. Cleavage of Bid into t-Bid represents a convergence point between the extrinsic and intrinsic pathways where BH3-only proteins are activated leading to inhibition of pro-survival proteins (Bcl-2/Bcl-XL) and activation of Bax/Bak. MOMP is induced either directly by Bax/Bak or through the formation of the mPTP (VDAC, ANT, CyD). Cytochrome c (Cyt C) is released from the mitochondria; the apoptosome (Cyt C, Apaf-1, caspase-9) is formed, leading to the activation of caspases, CADs, and the degradation of DNA. PM=plasma membrane. I acknowledge Aimee Winter for drawing the double helix DNA shown in the nucleus.
**Figure 1.2 Bcl-2 family members** Pro-survival Bcl-2 family members are depicted in blue, with transmembrane domain (TM) and Bcl-2 like homology domains (BH) 1-4. Pro-apoptotic family members with multiple domains are depicted in red (BH1-3 and TM), while BH3-only domain members are depicted in orange.
1.1.3 Apoptosis in Neurodegeneration

The means by which particular subsets of neurons degenerate in neurodegenerative diseases has been shown to rely upon apoptotic pathways. The pathology of Parkinson’s disease (PD) is due to the degeneration of dopamine neurons found in the substantia nigra located in the midbrain, where signs of apoptotic DNA damage have been observed in PD patients (Mochizuki et al., 1996). Substantia nigra samples from postmortem PD patients also display increased caspase-3, Bax, and apoptotic nuclei compared to age-matched control subjects (Hartmann et al., 2000; Tatton 2000). Furthermore, in PD models both in vitro and in vivo, inhibition of apoptotic proteins affords protection to degenerating dopamine neurons (Eberhardt et al., 2000). Indices of apoptosis have also been shown in amyotrophic lateral sclerosis (ALS), a disease in which the alpha motor neurons in the lumbar spinal cord degenerate. Motor neurons from spinal cord sections of ALS patients show decreased Bcl-2 mRNA but increased Bax mRNA levels compared to control subjects and non-neuronal tissue sections (Mu et al., 1996). An additional study showed an increase in pro-apoptotic protein levels (such as Bax) and a decrease in Bcl-2 protein levels at the mitochondria resulting in an increase in cytosolic Bcl-2 expression levels. Increased caspase-3 activation and apoptotic DNA damage were also observed (Martin 1999). Overall, the underlying mechanism of apoptosis induced during neuronal degeneration is hypothesized to be a consequence of mitochondrial dysfunction and oxidative stress, for which substantial evidence has been observed in these neurodegenerative diseases.
1.2 Mitochondrial Dysfunction and Oxidative Stress in Neurodegenerative Diseases

The high energy demands of the central nervous system (CNS) induce the dependence of neurons and glial cells on mitochondrial energy production. Mitochondria produce ATP from molecular oxygen using oxidative phosphorylation, a process that is driven by the ETC. Unfortunately; electron leakage from the ETC causes the formation of highly reactive species, such as superoxide. In order to combat the endogenous ROS produced at mitochondria, several free radical scavenging systems exist, including superoxide dismutase, thioredoxin, and glutathione (GSH). However, if ROS production exceeds the availability of the endogenous free radical scavengers, then indices of mitochondrial oxidative stress (MOS) accumulate, leading to the damage of mitochondrial lipids, DNA and proteins (Lin and Beal, 2006). It has been demonstrated in cardiac myocytes that ROS accumulation leads to mitochondrial membrane depolarization, a consequence dependent on cellular GSH concentrations. Furthermore, ROS stimulated mitochondrial membrane depolarization leads to ROS stimulated ROS release, inducing damage to surrounding mitochondria, propagating MOS signals, and enhancing cellular injury (Zorov et al., 2000; Zorov et al., 2006).

Mitochondrial dysfunction and subsequent oxidative stress are major contributing factors to many neurodegenerative diseases, such as ALS and PD. In particular, increased oxidative stress has been demonstrated through the depletion of GSH levels in postmortem studies of the substantia nigra of PD patients (Perry and Young, 1986). Furthermore, in an ALS mouse model, depleted levels of GSH within lumbar spinal cord tissue were correlated to the onset and progression of the ALS disease.
phenotype (Chi et al., 2007). Other indices of oxidative stress, such as damage to DNA, lipids and proteins are also prevalent in these neurodegenerative diseases. For example, sporadic ALS and PD patients have increases in oxidized Coenzyme Q10 and 8-hydroxy-2’-deoxyguanosine (8-OHdG; oxidative modification to DNA) in cerebral spinal fluid (CSF) samples (Murata et al., 2008; Isobe et al., 2010). Mitochondrial dysfunction is shown through decreased protein levels of complex II, III, and V in mitochondria from postmortem sporadic PD brain (Arthur et al., 2009). It is also well established that PD patients have decreased complex I activity, within postmortem samples of the substantia nigra (Schapira et al, 1990). The G93A mutant SOD1 mouse model of ALS displays aggregation of mitochondria and reduced activity of complexes I, II and IV in spinal cord tissues (Jung et al., 2002; Sotelo-Silveira et al., 2009). Furthermore, ALS patients also have mitochondrial aggregates in spinal cord anterior horn sections, and decreased cytochrome c oxidase activity (Sasaki and Iwata, 1996; Borthwick et al., 1999). The involvement of oxidative stress and mitochondrial dysfunction within neurodegeneration has been reviewed extensively (Lin and Beal, 2006; Federico et al., 2012).

1.3 Glutathione synthesis, functions and metabolism

Glutathione (GSH) is the most prominent cellular thiol with numerous functions and a distinct synthesis pathway. This tri-peptide consisting of glutamate, cysteine and glycine is present at higher concentrations than all other known free radical scavenging systems. As discussed above, depletion of GSH has been observed in several neurodegenerative diseases, and therefore its significance in neuronal survival is of interest. Here, its synthesis, functions, regulation and metabolism are reviewed.
1.3.1 Glutathione synthesis

The synthesis of GSH is limited to the cytosol and occurs in two ATP-dependent reactions (Griffith and Meister, 1985). The first step in GSH synthesis requires L-glutamate to react with ATP, leading to the formation of L-glutamylphosphate, a reaction intermediate, which then combines with L-cysteine to form γ-L-glutamyl-L-cysteine, ADP, and phosphate (Griffith, 1999; Forman et al., 2009; Figure 1.3). This first step in GSH synthesis is catalyzed by glutamate cysteine ligase (GCL; also known as γ-glutamylcysteine ligase) and occurs through the γ-carboxyl group of glutamate leading to the formation of a γ-peptidic linkage with the amino group of cysteine (Griffith, 1999; Wu et al., 2004). This γ-carboxyl linkage renders GSH uncleavable by all cellular proteases, except for γ-glutamyltranspeptidase (Wu et al., 2004; Lu, 2009). The second step in GSH synthesis is catalyzed by glutathione synthetase (GS), and begins with γ-L-glutamyl-L-cysteine reacting with ATP, to form the reaction intermediate γ-L-glutamyl-L-cysteinylphosphate, which then combines with L-glycine to form γ-L-glutamyl-L-cysteinyl-L-glycine or GSH, ADP and phosphate (Griffith, 1999; Forman et al., 2009). GCL is considered the rate limiting enzyme in GSH synthesis based on data showing that its overexpression, but not that of glutathione synthetase (GS), leads to an increase in cellular GSH content (Forman et al., 2009). The bioavailability of cysteine is also a rate limiting determinant for GSH synthesis (Griffith, 1999; Lu, 2009).
Figure 1.3 Glutathione Synthesis  

L-glutamate and L-cysteine are substrates for \( \gamma \)-glutamylcysteine ligase (GCL) in the formation of L-\( \gamma \)-glutamylcysteine. Next, L-glycine and L-\( \gamma \)-glutamylcysteine are substrates for glutathione synthetase (GS) in the formation of \( \gamma \)-glutamyl-cysteine-glycine, or GSH. The hydrolysis of ATP into ADP and phosphate is a requirement for each enzymatic step in GSH synthesis.
1.3.2 Regulation of glutathione synthesis

The regulation of GSH synthesis is modulated by several factors including; feedback inhibition, the availability of amino acid precursors, and post-translational modifications. The GCL holoenzyme has two subunits, GCLC (catalytic) and GCLM (modulatory). GCLC contains all of the enzymatic activity for the holoenzyme while GCLM regulates the holoenzyme (Huang et al., 1993). The GCL holoenzyme forms an intermolecular disulfide bond between the GCLC and GCLM subunits which increases the affinity of GCLC for glutamate under oxidizing conditions (Soltaninassab et al., 2000; Filomeni et al., 2002). However, when cellular GSH levels are increased, GSH reduces the intermolecular disulfide bond, decreasing the affinity of GCLC for glutamate, thus allowing for feedback inhibition of GCLC by GSH (Soltaninassab et al., 2000; Filomeni et al., 2002).

Second, the availability of amino acid precursors, particularly cysteine, affects the rate of GSH synthesis, and therefore, contributes to its regulation. Amino acid availability and preference for use in GSH synthesis is dependent on cellular activities and cell type. For example, the availability of specific precursors in the liver is dependent on several factors such as diet, amino acid transport, and the transsulfuration pathway. In liver, the major sources of cysteine are either provided from the metabolism of methionine, in which transsulfuration converts homocysteine to cysteine or through protein degradation (i.e. diet) (Griffith, 1999; Wu et al., 2004; Lu, 2009). Previous studies do not provide support for the use of the transsulfuration pathway within the brain, based on low levels of enzymatic activity and RNA expression of its constituents, and the
inability of methionine to increase GSH levels (Dringen and Hamprecht, 1999; Ishii et al., 2004). Overall, most tissues receive the precursors L-cysteine or L-cystine (disulfide form of cysteine) from GSH excreted into the plasma from the liver; and amino acid transport systems are important for maintaining cellular GSH in tissues other than liver (Griffith, 1999). In particular, the Na$^+$ independent $X_c^-$ amino acid transporter normally exchanges intracellular glutamate for extracellular cystine such that elevated extracellular glutamate concentrations can reduce the availability of cystine by inhibiting its transport through this system (Griffith, 1999; Lu, 2009). In the brain, high extracellular concentrations of glutamate and glycine are not present because these molecules function as neurotransmitters. Therefore, GSH precursors are effectively transported into neurons after their release from astrocytes (Dringen and Hirrlinger, 2003). Astrocytes export approximately 10% of their total intracellular GSH within one hour, and the GSH exported is broken down by $\gamma$-glutamyltranspeptidase and aminopeptidase N in order to provide precursors to neurons for de novo GSH synthesis (Dringen et al., 1997; Dringen et al., 1999; Dringen et al., 2001). Furthermore, differences in the precursors used between astrocytes and neurons have been documented. Astrocytes use cystine and glutamate as precursors transported through the $X_c^-$ amino acid transporter, while neurons use cysteine and glutamine for GSH precursors transported by excitatory amino acid transporters (EAAT) $X_{AG^-}$, these differences have been hypothesized to prevent competition for precursors between neighboring cells (Kranich et al., 1996; Dringen et al., 1999; Shanker et al., 2001; Dringen and Hirrlinger, 2003).
Finally, post-transcriptional and post-translational modifications of GCLC and GCLM also contribute to the regulation of GSH synthesis; however these effects have not been elucidated for GS (Lu, 2009). For both subunits of the holoenzyme, GCL, mRNA stabilization has been reported under oxidative conditions (Liu et al., 1998). GCLC phosphorylation and S-nitrosylation (at the active site thiol) has been shown to decrease its activity (Sekhar and Freeman, 1999; Griffith, 1999). During apoptosis, caspase-3 dependent cleavage of GCLC results in decreased activity (Franklin et al., 2003). Overall, the post-transcriptional and post-translational modifications of GCLC are the best characterized.

GCLC contains binding sites for the transcription factors AP-1, AP-2, NF-κB, SP-1 and ARE/EpRE (antioxidant response element/ electrophile response element); GCLM transcriptional binding sites have been less studied, but include AP-1 and ARE/EpRE (Moinov and Mulcahy, 1998; Griffith 1999). In addition, Nrf1 and Nrf2 can increase the transcription of GCLC by modulating AP-1 and NF-κB (Yang et al., 2005). NF-κB can regulate the transcription of GCLM through AP-1 (Yang et al., 2005). Finally, numerous oxidative stress inducers have been shown to increase either the transcription of GCLC or GCLM and in some cases both, however this appears to be tissue specific, and differences have been found between in vitro and in vivo studies (reviewed in Griffith, 1999 and Lu, 2009). GS transcription is regulated by NF-κB, AP-1, Nrf1 and Nrf2 (Yang et al., 2002; Yang et al., 2005; Lu, 2009).
1.3.3 Functions of glutathione

The functions of GSH are as diverse as its cellular compartmentalization; it has been shown to be present in the mitochondria, endoplasmic reticulum (ER), peroxisomes, nucleus and cytosol. Therefore, GSH functions as more than an antioxidant; it has been implicated in the control of the cell cycle, cell signaling, a store of cysteine, protein folding, the detoxification of xenobiotics in addition to other functions, all of which are reviewed below (Lu, 2009).

Within the mitochondrial matrix, GSH serves as an antioxidant, a function which is linked to other enzymatic systems of the mitochondria. GSH functions in conjunction with superoxide dismutase (SOD) to scavenge superoxide, where SOD converts superoxide into water and hydrogen peroxide (H$_2$O$_2$) while GSH works to detoxify the H$_2$O$_2$ produced. The reaction between H$_2$O$_2$ and GSH can occur both enzymatically and non-enzymatically. Glutathione peroxidase (GPx) is a selenoenzyme which facilitates the reduction of H$_2$O$_2$ into water, using GSH as an electron donor, forming glutathione disulfide (GSSG) (Meister and Anderson 1983; Dringen et al., 2000; Lu 2009). Next, glutathione reductase (GR) recycles GSSG at the expense of NADPH, into two GSH molecules (Meister and Anderson 1983; Dringen et al., 2000; Lu 2009; Figure 1.4). GSH can also scavenge the hydroxyl radical, peroxynitrite, and nitric oxide (Sjöberg et al., 1982; Koppal et al., 1999; Yadav and Mishra 2013). Other antioxidant functions of GSH include the reduction of lipid hydroperoxides and prevention of the fenton reaction from occurring between metals (copper and iron) and oxygen (Filomeni et al., 2002). The mechanism by which GSH prevents the fenton reaction has been hypothesized to rely
upon its role in the detoxification of copper and cellular trafficking of copper (Ferreira et al., 1993; Maryon et al., 2013).

Beyond its capacity as an antioxidant, GSH serves to detoxify certain cellular components and by-products. Glutathione-S-transferase (GST) facilitates the conjugation of GSH with proteins such as xenobiotics (quinones) and HNE (lipid radicals) (Meister, 1988). The addition of GSH to these toxic compounds induces their export to the extracellular matrix, where they are then metabolized by γ-glutamyltranspeptidase leading to the formation mercapturic acids and thus detoxifying these components (Boyland and Chasseaud, 1969; Dickinson and Forman, 2002; Lu, 2009). Post-translational modifications and protein conjugation are relevant to the cell signaling function of GSH. For example, GSH will conjugate with NO to form S-nitrosoglutathione (GSNO), which can participate in S-nitrosylation, form glutathione adducts with cysteine residues of proteins, or serve as a cellular NO reservoir and targeting (Ji et al., 1999; Andre and Felley-Bosco, 2003). In particular, GSNO has been shown to act as a neuroprotective agent during oxidative stress conditions (Rauhala et al., 1998). In addition, GST assisted GSH conjugation to proteins can either lead to the activation or loss of function of proteins due to structural changes (Filomeni et al., 2002).

In the ER, GSH is present in its oxidized form, GSSG, where it is necessary for protein folding and the formation of disulfide bonds within nascent proteins (Hwang et al., 1992; Mari et al., 2009). GSH is also important for the regulation of intracellular Ca$^{2+}$ levels, in which the inhibition of GSH synthesis and thus depleting cellular GSH levels caused an increase in intracellular Ca$^{2+}$ levels and a decrease in PC12 cell viability.
(Jurma et al., 1997). GSH has also been shown to protect the protein sulfhydryl groups on Ca\textsuperscript{2+} transporters from oxidation (Rimpler et al., 1999).

Nuclear localized GSH has a role in DNA synthesis and cell cycle control. Glutaredoxin is an enzyme which utilizes GSH as a reducing mechanism for ribonucleotide reductase (the rate limiting enzyme in DNA synthesis) which converts nucleotide diphosphates to deoxyribonucleotide disphosphates, an essential step in DNA synthesis (Holmgren, 1981; Pallardó et al., 2009). When GSH is depleted, it has been shown to inhibit DNA synthesis further implicating the requirement for GSH in this process (Dethlefsen et al., 1988; Pallardó et al., 2009). Inhibition of GSH synthesis and subsequent reductions in cellular GSH content induces a decrease in telomerase activity, as well as, decreased expression of the cell cycle regulatory proteins Id2 and E2F4 (Borrás et al., 2004). In addition, inhibition of GSH synthesis leads to cell cycle arrest, consistent with several studies that have shown GSH is required for cell cycle entry into the S phase (Shaw and Chou 1986; Messina and Lawrence 1989; Poot et al., 1995; Lu, 2009).

GSH also has CNS specific functions as shown in recent studies which have provided evidence that GSH may play a role in synaptic transmission, through an interaction with the glutamatergic receptors, NMDA and AMPA (Varga et al., 1997). The consequences of GSH binding to NMDA receptors in neurons is dependent on the concentration of GSH. For example, low concentrations of GSH (μM) prevent the binding of excitatory agonists, while high concentrations of GSH (mM) modulate redox sites of the NMDA receptor, increasing its currents (Janáky et al., 1999).
GSH appears to bind currently non-identified receptors, a phenomenon linked to sodium. However, the importance of these findings is limited due to the variability of findings and relatively few published studies (Janáky et al., 1999; Oja et al., 2000).

Finally, GSH affords protection from apoptotic stimuli. This function could be a culmination of its numerous other actions discussed above. It has been proposed that one of the earliest events in the induction of apoptosis is GSH depletion (Esteve et al., 1999; Franco et al., 2007). GSH depletion in the nucleus has been shown to increase cell susceptibility to DNA alkylating agents (Britten et al., 1991). Furthermore, GSH depletion in the cytosol and more specifically in the mitochondria has been shown to play a role in the apoptotic pathway, at the level of mitochondrial permeability transition (Armstrong and Jones, 2002). Mitochondrial DNA oxidation has been shown to correlate with a decrease in GSH and an increase in oxidized GSH levels, while total cellular GSH depletion is an event which occurs prior to DNA fragmentation (Esteve et al., 1999). These studies indicate that mitochondrial GSH plays an important role in cell survival, which is discussed in the next section.
Figure 1.4 Glutathione detoxification of $H_2O_2$ Superoxide ($O_2^-$) is dismutated into hydrogen peroxide ($H_2O_2$) and water ($H_2O$) via superoxide dismutase (SOD). $H_2O_2$ is broken down into $H_2O$ at the expense of two GSH molecules using the enzyme GSH peroxidase (GPx), forming oxidized GSH, or GSH disulfide (GSSG). GSH reductase (GR) uses NADPH as an electron donor to form two GSH molecules from GSGG, leading to the formation of NADP$^+$. Glucose-6-phosphate dehydrogenase (G6PD) then cycles NADP$^+$ back into NADPH.
1.4 The discrete mitochondrial glutathione pool is critical for cell survival

Although the mitochondrial GSH pool only constitutes 10-30% of total cellular GSH (percentage is specific to cell type); the concentration of GSH in the mitochondria is comparable to the cytosolic concentration of GSH which is ~1-10 mM (Smith et al., 1996; Filomeni et al., 2002; Circu et al., 2008). Mitochondria contain several antioxidant enzyme scavenging systems; however, GSH is present at a concentration that is 500-1000 times that of other antioxidant systems (Schafer and Buettner, 2001; Filomeni et al., 2002). Furthermore, mitochondria from brain rely upon GSH detoxification of matrix-generated H$_2$O$_2$, because catalase has only been found to be present on the outer mitochondrial membrane (Vitorica et al., 1984; Mari et al., 2009). The specific depletion of mitochondrial GSH in cerebellar granule neurons (CGNs), led to increased ROS production, mPTP induction, and cell death. In the same system, depletion of the cytosolic GSH pool did not recapitulate these effects (Wüllner et al., 1999). Furthermore, specific depletion of mitochondrial GSH in astrocytes sensitized these glial cells to cell death induced by either oxidative stress (H$_2$O$_2$) or nitrosative stress (SIN-1 or SNAP) (Muyderman et al., 2007). The importance of mitochondrial GSH has not just been demonstrated in the brain, but also in hepatocytes. For example, depletion of total cellular GSH (both mitochondrial and cytosolic) led to an increase in cell death and lipid peroxidation which was greater than when only the cytosolic pool of GSH was depleted (Meredith and Reed, 1982; Meredith and Reed, 1983). Several models of cell death lead to a specific decrease of mitochondrial GSH; and thus depletion and oxidation of this reservoir has been hypothesized to be the mode of cell toxicity. These studies include
toxicity induced by aromatic hydrocarbons in rat adrenal cells, hypoxia in hepatocytes, cisplatin treated renal cells and cardiomyocyte apoptosis in streptozotocin induced diabetes in rats and mice (Hallberg and Rydström 1989; Santos et al., 2003; Lluis et al., 2005; Ghosh et al., 2005; and Santos et al., 2007). Studies on the exact contribution of the mitochondrial GSH pool to cell survival have been relatively limited because the understanding of how to discretely manipulate this pool of GSH was not previously available. The recent identification of specific mitochondrial GSH transporters (discussed below) will now allow for more specific depletion of mitochondrial GSH pools and thus a better understanding of the involvement that this specific pool of GSH plays in cell survival can be elucidated. Nonetheless, current evidence suggests that the mitochondrial GSH pool is key to cell survival and resistance to apoptosis in numerous models.

1.5 Deciphering the mechanism by which glutathione is compartmentalized into mitochondria

The transport of GSH into mitochondria first became of interest to researchers in the late 1980s. At that time liver and kidney tissues were used primarily due to their high mitochondrial content. The first study completed by Griffith in 1985 demonstrated that mitochondria lack the enzymes required for the synthesis of GSH (Griffith and Meister, 1985). Moreover, the charge of GSH at physiological pH (-1 to -2), the relative negative charge of the mitochondrial membrane space in relation to the cytoplasm, and the similar mitochondrial and cytoplasmic GSH concentrations, do not allow for simple diffusion to account for the observed mitochondrial GSH levels (Lash, 2006). These studies suggested that either an active and/or facilitated process contributed to the movement of
GSH into mitochondria, and two inner membrane anion carriers, dicarboxylate (DIC, \textit{Slc25a10}) and 2-oxoglutarate (OGC, \textit{Slc25a11}) carriers, were subsequently identified as mitochondrial GSH transporters in both liver and kidney. These carriers belong to a mitochondrial super family, the solute carrier family that exhibit similar predicted 3D structure, namely, this family is characterized by containing three transmembrane domains and are believed to exist as homodimers (Lash, 2006). OGC is known to transport 2-oxoglutarate in exchange for dicarboxylates, while DIC exchanges dicarboxylates in exchange for phosphate. OGC functions to provide substrates for several metabolic processes including the citric acid cycle, malate/aspartate shuttle, 2-OG/isocitrate shuttle, nitrogen metabolism, and gluconeogenesis from lactate (Lash, 2006). DIC supplies dicarboxylates to the citric acid cycle, while also transporting thiosulfates into the cytoplasm for the enzymes rhodanese and thiosulfate reductase (Lash, 2006). The early studies and identification of these carriers as mitochondrial GSH transporters are reviewed below.

In the early studies regarding mitochondrial GSH transport, it was shown that there were two components, high-affinity and low-affinity in rat liver mitochondria, and both were inhibited by protonophores, glutamate, and the GSH analog, ophthalmic acid (Mårtensson et al., 1990). Respiratory rates of mitochondria were also shown to affect mitochondrial GSH transport in liver, while protonophores and antimycin A reduced uptake of GSH (Kurosawa et al., 1990). Collectively, these early studies allowed for the prediction that mitochondrial GSH transport may be determined by mitochondrial energy dependency.
Lash and colleagues were the first group to examine the potential contributions of previously identified electro-neutral anion carriers to mitochondrial GSH transport. Several molecules were found to inhibit GSH transport into isolated rat kidney mitochondria, including glutamate, malate/butylmalonate, succinate/phenylsuccinate, and phosphoenylpyruvate. In addition, GSH transport was also shown to be dependent on phosphate and ATP production (Chen and Lash, 1998). These studies confirmed that both the DIC and OGC were able to transport GSH into mitochondria (Chen and Lash, 1998). The tricarboxylate carrier was ruled out, because its inhibitor, phosphoenylpyruvate induced mitochondrial permeability transition, and thus decreased mitochondrial GSH transport via a non-specific mechanism (Chen and Lash, 1998). Further investigation of the effect glutamate exhibited on GSH transport into isolated mitochondria, ruled out the involvement of the glutamate and glutamate/aspartate transporters in this process. It was shown that GSH did not inhibit glutamate transport, that GSH transport was independent of pH, and aspartame did not affect GSH transport. Therefore, it was concluded that the glutamyl residue was important for GSH transport, thus elucidating the effect of glutamate on GSH transport (Chen and Lash, 1998).

In a separate study, fractions enriched from rabbit kidney mitochondria were reconstituted into proteoliposomes to study GSH transport mechanisms. One study reported that, DIC and OGC were responsible for approximately 70-80% of mitochondrial GSH transport in kidney (Chen et al., 2000). Finally, overexpression of OGC or DIC in NRK-52E rat kidney cells, led to an increase in mitochondrial GSH transport rates and increased resistance to apoptosis (Lash et al., 2002; Xu et al., 2006).
Identification of DIC and OGC mediated GSH transport in kidney made a profound impact on this field of research, and stimulated interest of mitochondrial GSH transport mechanisms in other tissue types. Studies completed in isolated rat liver mitochondria showed that GSH transport was only partially inhibited by phenylsuccinato and butylmalonate, and was independent of ATP hydrolysis (Zhong et al., 2008). Furthermore, overexpression of OGC in H4IIE rat hepatoma cells, led to a significant increase in mitochondrial GSH uptake, and resistance to several apoptotic stimuli (Zhong et al., 2008). Overall, mitochondrial GSH transport in liver may only be partially dependent on OGC and DIC (approx. 45-50%), and therefore similar to kidney, other carriers could play a role in this process (Lash, 2006).

Mitochondrial GSH transport mechanisms may vary among tissues and cell types for several reasons. For example, although DIC has been found to have consistent expression and amino acid sequence homology among various tissues and species, OGC does not display the same consistency (Lash, 2006). Specifically, there are six amino acid differences between kidney and brain OGC constructs, two of which are different in charge or polarity, and three of which are in close proximity to transmembrane domains (Lash, 2006). OGC mRNA levels are highest in heart, skeletal muscle, brain, liver and kidney, but low in lung and pancreas. Consistent with this finding, expression of mitochondrial carriers is dependent on the energy demand of the cell type (Lash, 2006). Therefore, mitochondrial GSH transport mechanisms appear to be tissue and cell type specific.
More recent studies have emerged examining mitochondrial GSH transport in the brain. Mitochondrial GSH transport in isolated mitochondria from whole rat brain was shown to be inhibited by the substrates citrate, isocitrate, malate, and 2-oxoglutarate. In the same system, the inhibitors of specific transporters, butylmalonate (DIC), phenylsuccinate (OGC), and benzene 1,2,3-tricarboxylate (tricarboxylate carrier, TCC) significantly prevented the uptake of GSH into isolated mitochondria (Wadey et al., 2009). The authors of this particular study concluded that isolated rat brain mitochondria were dependent on TCC for mitochondrial GSH transport, as butylmalonate (specific DIC inhibitor) and phenylsuccinate (specific OGC inhibitor) had previously been shown to have a weak inhibitory effect on TCC. However, the authors failed to explain the effects of the substrates malate (DIC substrate) and 2-oxoglutarate (OGC substrate) on mitochondrial GSH uptake within this study (Wadey et al., 2009). While cortical neurons and astrocytes express both OGC and DIC, it appears that isolated mitochondria from the cortex depend primarily on DIC for GSH transport (Kamga et al., 2010). Interestingly, inhibition of DIC-mediated GSH transport in isolated mitochondria led to an increase in ROS and H$_2$O$_2$ production, impaired complex I activity, and decreased mitochondrial membrane potential (Kamga et al., 2010). Finally, PC12 cells (rat adrenal pheochromocytoma cell line) were also found to be dependent on DIC, but not OGC, for mitochondrial GSH transport (Kamga et al., 2010). Overall, studies examining mitochondrial GSH transport mechanisms and mitochondrial GSH transport regulation in brain are limited and warrant further investigation.
1.6 Past studies linking Bcl-2 overexpression to cellular glutathione metabolism

1.6.1 The diverse functions of Bcl-2

Beyond its functions in inhibiting pro-apoptotic proteins, Bcl-2 is also involved in the regulation of the cell cycle and Ca\textsuperscript{2+} homeostasis. These additional functions of Bcl-2 in promoting cell survival were discovered as a consequence of numerous studies examining Bcl-2-dependent protection from a variety of toxic insults (Table 1.1). Bcl-2 was found to protect from both free radical generators and toxic insults which lead to cell death independent of free radicals or reactive intermediates (Table 1.1). However, the protection Bcl-2 afforded from agents which act through Ca\textsuperscript{2+} mediated cell stress, was found to rely upon the targeting of Bcl-2 to the endoplasmic reticulum (ER) and the interaction between Bcl-2 and the inositol 1, 4, 5-trisphosphate (IP3) receptor. Previous work indicated that the BH4 domain of Bcl-2 was responsible for interacting with and inhibiting Ca\textsuperscript{2+} release from the endoplasmic reticulum (ER) via the IP3 receptor (Rong et al., 2009). Overexpression of Bcl-2 was found to maintain ER Ca\textsuperscript{2+} uptake and inhibit Ca\textsuperscript{2+} efflux, thus potentiating apoptosis induced by thapsigargin, an inhibitor of the ER Ca\textsuperscript{2+} ATPase (He et al., 1997). Furthermore, Bcl-2 was also shown to regulate mitochondrial Ca\textsuperscript{2+} uptake in a GT1-7 neuronal cell line, thus preventing mitochondrial Ca\textsuperscript{2+} overload, mitochondrial permeability transition, and apoptosis (Murphy et al., 1996). Bcl-2 also functions as a repressor of the cell cycle transition between G1 to S phase, a phenomenon that is hypothesized to be dependent on the regulation of ROS by Bcl-2 (Deng et al., 2003). The mechanism by which Bcl-2 suppresses cell cycle transition was found to be dependent on increased expression of p27 and p130; both of which are
repressors of the E2 family of transcription factors (E2F4; E2F1) which control the expression of critical cell cycle proteins (Vairo et al., 2000). Interestingly, the functions that Bcl-2 contributes to cell survival are similar to the previously identified functions of GSH discussed above (i.e. cell-cycle, prevention of apoptosis, and Ca\(^2+\) signaling). Thus, it is not surprising that Bcl-2 expression and GSH have been related in several ways, as discussed below.
### Table 1.1 Bcl-2 protects from a variety of insults in diverse systems

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Insult</th>
<th>Citation</th>
</tr>
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<tbody>
<tr>
<td>PC12</td>
<td>Growth factor withdrawal, A23187, Dopamine, Serum Withdrawal, H₂O₂, Aβ₂₅₋₃₅, FeSO₄, HNE, AMVN, Cycloheximide, Actinomycin D, colchicine, EGTA, Enediyne-5, cisplatin</td>
<td>Mah et al., 1993&lt;br&gt;Sato et al., 1994&lt;br&gt;Lindenboim et al. 1995&lt;br&gt;Cortazzo and Schor 1996&lt;br&gt;Ellerby et al., 1996&lt;br&gt;Offen et al., 1997&lt;br&gt;Kruman et al., 1997&lt;br&gt;Tyurina et al., 1997&lt;br&gt;Bruce-Keller et al., 1998&lt;br&gt;Jang and Surh et al., 2004</td>
</tr>
<tr>
<td>GT1-7</td>
<td>Potassium cyanide with glucose deprivation, BSO, Diethyl maleate, Ethacrylic acid, Serum Withdrawal</td>
<td>Myers et al. 1995&lt;br&gt;Kane et al., 1993/1995&lt;br&gt;Ellerby et al., 1996</td>
</tr>
<tr>
<td>Hippocampal neurons</td>
<td>Adriamycin, Glutamate, Glucose deprivation</td>
<td>Lawrence et al. 1996</td>
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<tr>
<td>L929 cells</td>
<td>TNF</td>
<td>Hennet et al., 1993</td>
</tr>
<tr>
<td>Hematopoietic</td>
<td>IL-3 withdrawal</td>
<td>Baffy et al., 1993</td>
</tr>
<tr>
<td>CSM14.1</td>
<td>Serum withdrawal, A23187, Glucose deprivation, menadione, t-BOOH, BSO</td>
<td>Zhong et al., 1993</td>
</tr>
<tr>
<td>Thymocytes</td>
<td>Dexamethasone, radiation, anti-CD3</td>
<td>Sentman et al., 1991</td>
</tr>
<tr>
<td>Hippocampal neurons</td>
<td>NGF withdrawal-delay in apoptosis</td>
<td>Garcia et al., 1992</td>
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<tr>
<td>LY-ar</td>
<td>Gamma-radiation</td>
<td>Mirkovic et al., 1997</td>
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<tr>
<td>Transgenic mice</td>
<td>BSO-unweaned pups, adriamycin, focal ischemia, 3-nitropropionic acid, permanent ischemia induced by middle cerebral artery occlusion, natural occurring cell death</td>
<td>Martinou et al. 1994&lt;br&gt;Lawrence et al. 1996&lt;br&gt;Merad-Saidoune et al., 1999&lt;br&gt;Bogdanov et al., 1999</td>
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<tr>
<td>Astrocytes</td>
<td>Glucose deprivation, H₂O₂</td>
<td>Papadopoulos et al., 1998</td>
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<td>FL15.12</td>
<td>IL-3 withdrawal, H₂O₂, menadione</td>
<td>Nunez et al., 1990&lt;br&gt;Hockenbery et al., 1990&lt;br&gt;Hockenbery et al., 1993</td>
</tr>
<tr>
<td>FDC-P1, 32D</td>
<td>IL-4, GM-CSF, IL-3 Withdrawal</td>
<td>Nunez et al., 1990</td>
</tr>
<tr>
<td>HL-60</td>
<td>CCCF, STS</td>
<td>Armstrong and Jones, 2002</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>H₂O₂, glucose withdrawal</td>
<td>Rimpler et al., 1999</td>
</tr>
</tbody>
</table>
1.6.2 Bcl-2 expression influences glutathione

The first evidence presenting an influence of Bcl-2 on GSH metabolism developed from early studies showing that its overexpression not only protected from oxidative stress but also increased cellular GSH levels. These studies, originally conducted to determine if the apoptotic pathway was conserved among various toxic insults, found that enhanced Bcl-2 expression provided protection from oxidative stress independent of inhibiting free radical generation (Hockenbery et al., 1993). A separate study determined that Bcl-2 overexpression afforded protection from apoptosis induced by GSH depleting agents such as, BSO, diethyl maleate, and ethacrynic acid, in a GT1-7 hypothalamic neural cell line (Kane et al., 1993). Finally, Bcl-2 overexpression in a variety of cell types leads to an increase in cellular GSH concentration (reviewed in Table 1.2). One particular study aimed to evaluate the cell type specific involvement of Bcl-2 expression on GSH metabolism (Schor et al., 2000). However, upon close examination of their data both cell types (PC12 and MCF-7) displayed increased basal cellular GSH content, but no differences in antioxidant enzyme activities were observed (Schor et al., 2000). After review of the literature regarding Bcl-2 overexpression and cellular GSH status, it was found that only one cell type out of nine, HL-60 (Human promyelocytic leukemia cells), did not show increased cellular GSH levels in the presence of increased Bcl-2 (Table 1.2., Armstrong and Jones, 2002). In addition, two separate studies examining HeLa cells showed a variation in the effects of Bcl-2 on cellular GSH levels; Voehringer et al. only showed increased GSH in the nuclear compartment where as
Meredith et al., showed a total cellular GSH increase (Meredith et al., 1998; Voehringer et al., 1998). These differences may be due to the different techniques used to overexpress Bcl-2, such as retroviral gene transfer (in HL-60 cells) and two distinct vectors used for stable transfection in the separate HeLa cell studies. Furthermore, the strength of the promoter used to drive expression could cause variation in results in the same cell line (Table 1.2). The most evident variable effect induced by the overexpression of Bcl-2 in different cell types, is its effect on antioxidant enzymes, as shown in Table 1.2. In vivo studies observed that knockout of Bcl-2 in mice led to a decrease in the activities of several antioxidant enzyme systems, suggesting that Bcl-2 may have multiple effects regarding cellular antioxidant status (Hochman et al., 1998). Overall, most studies which have examined the effects of Bcl-2 overexpression have correlated it with an increase in cellular GSH content (Table 1.2). A few possible mechanisms have been suggested in regards to how enhanced Bcl-2 expression leads to increased cellular GSH content. First, Bcl-2 overexpression in PC12 cells led to constitutive activation and enhanced transcriptional activity of NF-κB which led to downstream increases in GCLC levels (Jang and Surh, 2004). Therefore, Bcl-2 overexpression in this system appeared to induce an increase in de novo GSH synthesis. Second, enhanced Bcl-2 expression in HeLa cells did not alter the levels of GSH synthesizing enzymes, but inhibited methionine dependent GSH extrusion from cells at the plasma membrane, which correlated with an increase in cellular GSH, levels (Meredith et al., 1998). Finally, Bcl-2 overexpression in PC12 and GT1-7 cells led to an
increase in NADPH and decrease in NAD$^+$ levels, as well as a decrease in the ratio of ATP/ADP (Ellerby et al., 1996). If Bcl-2 overexpression induced an increase in NADPH production, this could lead to an increase in cellular GSH by the reduction of GSSG (see Figure 1.4). However, after examining flux through the pentose-phosphate pathway, it was concluded that Bcl-2 overexpression did not exert an effect on the production of NADPH (Ellerby et al., 1996).
Table 1.2 Effects of Bcl-2 overexpression on glutathione levels and antioxidant enzymes in various systems

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Effect on antioxidant enzymes</th>
<th>Effect on GSH</th>
<th>Mechanism of Bcl-2 expression</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC12</td>
<td>Increase in Cu/Zn SOD1 and GPx</td>
<td>Increase</td>
<td>Retroviral</td>
<td>Bruce-Keller et al., 1998</td>
</tr>
<tr>
<td>MCF-7</td>
<td>No change in GR, GSH synthesis</td>
<td>Increase</td>
<td>Plasmid/Stable</td>
<td>Schor et al., 2000</td>
</tr>
<tr>
<td>GT1-7</td>
<td>ND</td>
<td>Increase</td>
<td>Retroviral</td>
<td>Kane et al., 1993</td>
</tr>
<tr>
<td>PC12</td>
<td>Increase in SOD and CAT</td>
<td>Increase</td>
<td>Retroviral</td>
<td>Ellerby et al., 1996</td>
</tr>
<tr>
<td>GT1-7</td>
<td>Increase in SOD and GR</td>
<td>Increase</td>
<td>Retroviral</td>
<td>Ellerby et al., 1996</td>
</tr>
<tr>
<td>HeLa</td>
<td>ND</td>
<td>Increase</td>
<td>Tet-repressible promoter/Stable</td>
<td>Voehringer et al., 1998</td>
</tr>
<tr>
<td></td>
<td>ND</td>
<td>Increase</td>
<td>Plasmid/Stable</td>
<td>Meredith et al., 1998</td>
</tr>
<tr>
<td>LY-ar</td>
<td>ND</td>
<td>Increase</td>
<td>Endogenous</td>
<td>Mirkovic et al., 1997</td>
</tr>
<tr>
<td>HL60</td>
<td>ND</td>
<td>Decreased</td>
<td>Retroviral</td>
<td>Armstrong and Jones, 2002</td>
</tr>
<tr>
<td>Astrocytes</td>
<td>Increase in SOD and GSH Px</td>
<td>Increase</td>
<td>Retroviral</td>
<td>Papadopoulos et al., 1998</td>
</tr>
<tr>
<td>NT-2/D1 SK-N-MC</td>
<td>Increase in Cu/Zn SOD, CAT, GPx</td>
<td>Increase</td>
<td>Plasmid, stable</td>
<td>Lee et al., 2001</td>
</tr>
<tr>
<td>HeLa</td>
<td>ND</td>
<td>Increase</td>
<td>Stable transfection</td>
<td>Meredith et al., 1998</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>ND</td>
<td>Increase</td>
<td>Retroviral</td>
<td>Rimpler et al., 1999</td>
</tr>
</tbody>
</table>
The next major finding correlating an effect of Bcl-2 expression on GSH showed that cellular trafficking of Bcl-2 influences the cellular localization of GSH. Voehringer and colleagues observed that overexpression of Bcl-2 in HeLa cells caused a significant increase in nuclear Bcl-2 levels and a subsequent relocalization of GSH to the nucleus. In addition, the increased nuclear localization of Bcl-2 led to enhanced nuclear GSH transport (Voehringer et al., 1998). Therefore, the trafficking of Bcl-2 appears to influence the cellular compartmentalization of GSH. The major effects of Bcl-2 on cellular GSH are reviewed in Figure 1.5.

Further evidence linking Bcl-2 expression with GSH metabolism derived from cancer studies. It was found that cancer cells which overexpressed Bcl-2 (LY-ar; mouse lymphoma cells and HeLa cells) were highly resistant to gamma radiation-induced apoptosis. However, upon culturing these cells in cysteine/methionine free media or treatment with diethyl maleate which reduces GSH levels, resensitization of these cells to gamma-radiation induced apoptosis occurred (Mirkovic et al., 1997; Voehringer et al., 1998). In contrast, Bcl-2 overexpression in PC12 cells caused the chemotherapeutic drug, neocarzinostatin (NCS) to significantly increase apoptosis. NCS requires reduction by thiols in order to be activated and the increased GSH due to Bcl-2 overexpression could therefore account for the increased toxicity of NCS in these cells. Indeed, BSO pre-treatment prevented Bcl-2 from rendering the PC12 cells more susceptible to NCS and Bcl-2 conferred protection from both enedicine-5 and cisplatin, which are insensitive to cellular redox status (Cortazzo and Schor, 1996). Upon depletion of GSH in HL-60 cells
overexpressing Bcl-2, both the mitochondrial membrane potential and the ratio of GSH/GSSG were significantly decreased while ROS species production and apoptosis were increased compared to control cells (Armstrong and Jones, 2002).

More recently, it has been shown that the use of BH3 mimetics, which inhibit the pro-survival functions of Bcl-2, affect mitochondrial function and GSH. For example, in HeLa cells overexpressing Bcl-2, ABT-737 BH3-mimetic treatment leads to an increase in caspase-dependent apoptosis and ROS production, with a decrease in cellular GSH content (Howard et al., 2009). Furthermore, our lab has shown that inhibition of Bcl-2 using two distinct BH3 mimetics leads to mitochondrial oxidative stress, disruption of mitochondrial membrane potential, and displacement of GSH from mitochondria. Finally, Bcl-2 was shown to be a GSH binding protein, providing a unifying hypothesis for the antioxidant function of Bcl-2 and its effect on GSH metabolism in various systems (Zimmermann et al., 2007).

Based on the lack of studies examining the regulation of mitochondrial GSH transport and its involvement in neuronal survival, the major aims of this thesis are to provide novel data in these areas. Here it is shown that Bcl-2 is a novel interacting partner for OGC and this interaction is dependent on GSH. Furthermore, discrete inhibition of a mitochondrial GSH transporter (DIC) in primary neurons induces significant sensitization to both oxidative and nitrosative stress conditions. Conversely, stable overexpression of OGC induces specific increases in mitochondrial GSH and, strikingly, increased resistance to oxidative and nitrosative stress. Finally, increased mitochondrial GSH leads to an upregulation of Bcl-2 in stable OGC overexpressing cell
lines while knockdown of Bcl-2 decreases mitochondrial GSH levels and resensitizes these cells to oxidative stress. Therefore, Bcl-2 and OGC work in a synergistic manner in the transport of mitochondrial GSH in order to promote neuronal survival.
Figure 1.5 Overview of the effects of Bcl-2 on cellular GSH At the plasma membrane (PM) Bcl-2 inhibits methionine-dependent GSH efflux. Inhibition of Bcl-2 with HA14-1 leads to mitochondrial GSH extrusion, while also inhibiting the binding of Bcl-2 to GSH. Bcl-2 redistribution to the nucleus increases nuclear GSH content. Bcl-2 constitutively activates NF-κB causing an increase in GCLC levels and thus de novo GSH synthesis.
CHAPTER TWO: BCL-2 IS A NOVEL INTERACTING PARTNER FOR THE 2-OXOGLUTARATE CARRIER AND A KEY REGULATOR OF MITOCHONDRIAL GLUTATHIONE

2.1 Abstract

Despite making up only a minor fraction of the total cellular glutathione, recent studies indicate that the mitochondrial glutathione pool is essential for cell survival. Selective depletion of mitochondrial glutathione is sufficient to sensitize cells to mitochondrial oxidative stress (MOS) and intrinsic apoptosis. Glutathione is synthesized exclusively in the cytoplasm and must be actively transported into mitochondria. Therefore, regulation of mitochondrial glutathione transport is a key factor in maintaining the antioxidant status of mitochondria. Bcl-2 resides in the outer mitochondrial membrane where it acts as a central regulator of the intrinsic apoptotic cascade. In addition, Bcl-2 displays an antioxidant-like function that has been linked experimentally to the regulation of cellular glutathione content. We have previously demonstrated a novel interaction between recombinant Bcl-2 and reduced glutathione (GSH), which was antagonized by either Bcl-2 homology-3 domain (BH3) mimetics or a BH3-only protein, recombinant Bim. These previous findings prompted us to investigate if this novel Bcl-2/GSH interaction might play a role in regulating mitochondrial glutathione transport. Incubation of primary cultures of cerebellar granule neurons (CGNs) with the BH3 mimetic HA14-1 induced MOS and caused specific depletion of the mitochondrial glutathione pool. Bcl-2 was co-immunoprecipitated with GSH after chemical cross-
linking in CGNs and this Bcl-2/GSH interaction was antagonized by preincubation with HA14-1. Moreover, both HA14-1 and recombinant Bim inhibited GSH transport into isolated rat brain mitochondria. To further investigate a possible link between Bcl-2 function and mitochondrial glutathione transport, we next examined if Bcl-2 associated with the 2-oxoglutarate carrier (OGC), an inner mitochondrial membrane protein known to transport glutathione in liver and kidney. After co-transfection of CHO cells, Bcl-2 was co-immunoprecipitated with OGC and this novel interaction was significantly enhanced by glutathione monoethyl ester. Similarly, recombinant Bcl-2 interacted with recombinant OGC in the presence of GSH. Bcl-2 and OGC co-transfection in CHO cells significantly increased the mitochondrial glutathione pool. Finally, the ability of Bcl-2 to protect CHO cells from apoptosis induced by hydrogen peroxide was significantly attenuated by the OGC inhibitor phenylsuccinate. These data suggest that GSH binding by Bcl-2 enhances its affinity for the OGC. Bcl-2 and OGC appear to act in a coordinated manner to increase the mitochondrial glutathione pool and enhance resistance of cells to oxidative stress. We conclude that regulation of mitochondrial glutathione transport is a principal mechanism by which Bcl-2 suppresses MOS.
2.2 Introduction

Mitochondrial oxidative stress (MOS) plays a key role in the pathology underlying several neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), Parkinson’s disease, and Alzheimer’s disease (Lin and Beal, 2006). Accordingly, elucidating the pathways that regulate the mitochondrial oxidant/antioxidant balance is essential to develop novel therapeutics for neurodegeneration. Glutathione is an endogenous tripeptide antioxidant and a key player in averting MOS and evading apoptosis (reviewed in Franco and Cidlowski, 2009). It has been previously shown that selective depletion of mitochondrial glutathione sensitizes cells to oxidative or nitrosative stress (Muyderman et al., 2004; Muyderman et al., 2007). Moreover, glutathione depletion can induce apoptosis directly through opening of the mitochondrial permeability transition pore (Armstrong and Jones, 2002). In addition, γ-glutamylcysteine synthetase knockout mice, in which glutathione synthesis is inhibited and glutathione is depleted, display significant apoptotic cell death in multiple tissues (Dalton et al., 2004). These findings demonstrate that maintenance of cellular glutathione and, in particular, the mitochondrial glutathione pool is crucial for cell survival (Sims et al., 2004; Mari et al., 2009). Glutathione synthesis occurs exclusively in the cytosol because the enzymes required for its synthesis are absent within mitochondria (Griffith and Meister, 1985). Furthermore, glutathione has an overall negative charge at physiological pH and mitochondria exhibit a large negative membrane potential; consequently, glutathione transport into mitochondria cannot be explained by simple diffusion (Griffith and Meister, 1985; Lash, 2006). Previously, two
inner mitochondrial membrane (IMM) anion carriers were identified in kidney and liver as glutathione transporters, the 2-oxoglutarate carrier (OGC; *Slc25a11*) and the dicarboxylate carrier (DIC; *Slc25a10*) (Chen and Lash, 1998; Chen et al., 2000; Coll et al., 2003; Zhong et al., 2008). Overexpression of either OGC or DIC in a rat renal proximal tubular cell line (NRK-52E cells) significantly enhanced mitochondrial glutathione transport and protected these cells from chemically induced apoptosis, such as that induced by tert-butylhydroperoxide (Lash et al., 2002; Xu et al., 2006). In the context of the CNS, few studies have examined the mechanisms responsible for mitochondrial glutathione transport. In one study, glutathione transport into isolated rat brain mitochondria seemed to be influenced most by inhibitors of the tricarboxylate carrier rather than OGC or DIC (Wadey et al., 2009). However, another study showed that an inhibitor of DIC, butylmalonate, significantly decreased the glutathione content of isolated mouse brain mitochondria, suggesting that DIC may be the major glutathione transporter in mouse cerebral cortical mitochondria (Kamga et al., 2010). The authors of this study also showed that both OGC and DIC are expressed in cortical neurons and astrocytes. These studies suggest that multiple IMM anion transporters might be involved in mitochondrial glutathione transport in the CNS. Yet, essentially nothing is known about how the function of these IMM glutathione transporters is regulated. Previous studies have shown that the antiapoptotic protein Bcl-2 displays an antioxidant-like effect in response to either exogenous oxidative stress or glutathione depletion (Hockenbery et al., 1993; Kane et al., 1993). Overexpression of Bcl-2 leads to an increase in the cellular content of glutathione (Ellerby et al., 1996; Voehringer and Meyn, 2000). In contrast,
Bcl-2 knockout mice show reduced glutathione levels and glutathione peroxidase activity in brain tissue and demonstrate enhanced susceptibility to MOS-induced neuronal cell death (Hochman et al., 1998). Thus, the antioxidant-like function of Bcl-2 depends, in large part, on its potential to regulate the cellular glutathione status. In this context, we have previously shown that recombinant Bcl-2 is capable of directly binding to GSH in vitro, an interaction that is antagonized by the Bcl-2 homology-3 domain (BH3) mimetics HA14-1 and compound 6, as well as the BH3-only protein, Bim (Zimmermann et al., 2007). Interestingly, several BH3-only proteins are known to induce a pro-oxidant state at mitochondria, suggesting that disruption of this Bcl-2/GSH interaction might be an underlying factor in this effect (Ding et al., 2004; Liu et al., 2005). Collectively, these findings prompted us to hypothesize that Bcl-2 might be a key regulator of the mitochondrial glutathione pool. Here, we show that Bcl-2 interacts with GSH in intact primary cerebellar granule neurons (CGNs). As we have shown previously using recombinant proteins, this Bcl-2/GSH interaction is disrupted by the BH3 mimetic HA14-1. Consistent with a central role for Bcl-2 in maintenance of the mitochondrial glutathione pool, both HA14-1 and Bim inhibited mitochondrial GSH transport. Most significantly, in cotransfected CHO cells, Bcl-2 coimmunoprecipitates with OGC and this novel interaction is markedly enhanced by glutathione monoethylester (GSH-MEE). Moreover, Bcl-2 and OGC coexpression significantly increases the mitochondrial glutathione pool. Finally, we show that the ability of Bcl-2 to protect cells from apoptosis induced by hydrogen peroxide depends on OGC activity. We conclude that Bcl-2 is a novel interacting partner for OGC and a central regulator of the mitochondrial glutathione
pool. This newly discovered property of Bcl-2 suggests a molecular mechanism by which Bcl-2 protects cells from oxidative injury.

2.3 Materials and Methods

2.3.1 Materials

Ethacrynic Acid, Triton X-100, DTT, anti-tubulin antibody, DAPI, and phenylsuccinic acid were received from Sigma Aldrich (St. Louis, MO). HA14-1 was purchased from Alexis Biochemicals (Enzo Life Sciences, Plymouth Meeting, PA). Glutathione assay kit was obtained from Oxford Biomedical (Rochester Hills, MI). Mitochondrial Cytosolic fractionation kit was purchased from Biovision (Mountain View, CA). MDA assay kit was obtained from OxisResearch (Percipio Bioscience, Foster City, CA). Anti-Cox-IV was purchased from Cell Signalling (Beverly, MA). Glutathione monoethylester and rotenone were received from Calbiochem (San Diego, CA). Anti-V5 antibody was purchased from Abcam (Cambridge, MA). Recombinant BimL was obtained from R&D systems (Minneapolis, MN). The anti-GSH antibody and protein A/G beads were received from Santa Cruz Biotechnology (Santa Cruz, CA). Disuccinimidyl suberate (DSS) was purchased from Pierce Chemical (Rockford, IL). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA). Anti-Bcl-2 antibody was from BD Pharmigen (Franklin Lakes, NJ). Mouse TrueBlot® ULTRA Anti-Mouse Ig HRP secondary antibody was obtained from eBioscience (San Diego, CA). GST tagged Slc25a11 (GST-OGC) recombinant protein and Slc25a11 (OGC) antibody were purchased from Novus Biologicals (Littleton, CO). Recombinant Bcl-2 protein was obtained from Calbiochem (Darmstadt, Germany). Optimem media was purchased from
Gibco (Carlsbad, CA). Maxiprep Kit was obtained from Qiagen (Valencia, CA). Anti-active caspase-3 was purchased from Promega (Madison, WI). Fitc secondary antibody for immunohistochemistry was from Jackson ImmunoResearch Inc (West Grove, PA). ECL, Percoll, and secondary antibodies for immunoblotting were purchased from GE Life Sciences (Piscataway, NJ). V5-OGC plasmid was a generous gift from Dr. Lash, Wayne State University (Detroit, MI). The Bcl-2 plasmid was a generous gift from Dr. Hardwick, Johns Hopkins University (Baltimore, MD).

2.3.2 Cerebellar Granule Neuron (CGN) Culture

CGNs were isolated from P7 Sprague Dawley rat pups and cultured as previously described (Zimmerman et al., 2007). All animal manipulations were performed in accordance with and under approval of the University of Denver Institutional Animal Care and Use Committee.

2.3.3 Immunohistochemistry

CGNs or CHO were transfected and/or treated as described under the Figure Legends section and fixed with 4% paraformaldehyde. Next, cells were blocked and permeabilized in PBS, pH 7.4 with 5% BSA and 0.2% Triton X-100 for 1 h, followed by incubation with the primary antibody overnight at 4°C diluted in 0.2% Triton X-100 and 2% BSA in PBS. After which, the primary antibody was removed and cells were washed 5X with PBS over 30 min. Next, the cells were incubated with secondary antibody and DAPI for 1 h at room temperature, diluted in 0.2% Triton X-100 and 2% BSA in PBS. Cells were washed 5X with PBS over 30 min, and placed in anti-quench. Images were captured using a Zeiss Axioplan 2 fluorescence microscope equipped with a Cooke
Sensicam CCD camera and Slidebook Image analysis software (Intelligent Imaging Innovations, Inc Denver, CO).

2.3.4 CHO cell culture

K1-CHO (Chinese Hamster Ovary) cells were plated on 35-mm diameter plastic dishes in Ham’s F12 media containing 10% fetal bovine serum, 2 mM L-glutamine and (100 U/mL/100 μg/mL) penicillin/streptomycin. Cells were cultured overnight at 37°C in 10% CO₂. The following day cells were prepared for transfection or treatment, at which point cultures were 60–80% confluent.

2.3.5 Plasmid Preparation

DsRed2, Bcl-2, and V5-OGC plasmids were transformed using 50 ng of plasmid in JM109 Escherichia coli (E. Coli) cells, and grown on LB agar plates containing 35 μg/mL kanamycin sulfate or 100 μg/mL ampicillin sodium salt at 37°C overnight. Starter cultures were grown in LB broth with 35 μg/mL kanamycin sulfate or 100 μg/mL ampicillin sodium salt for 8 h at 37°C, and diluted 1:250 into overnight cultures, for plasmid purification using the Qiagen Maxi Prep Kit (Valencia, CA). DNA concentrations were determined using a ThermoScientific NanoDrop 2000.

2.3.6 Transfection

DsRed2 (Con), Bcl-2, or V5-OGC were used at a concentration of 5 μg/mL. Plasmids were transfected using a standard Lipofectamine 2000 protocol. CHO cells cultures were incubated with the plasmid/Lipofectamine 2000 mixture in Opti-MEM, for 6 h at 37°C and 10% CO₂. Transfection media was removed and cell cultures were placed

45
in 1 mL of Ham’s F12 media and incubated overnight at 37°C and 10% CO₂. Indicated treatments were administered 24 h post-transfection.

2.3.7 Mitochondrial/Cytosolic Fractionation

Cells were treated as indicated in the “Results” or “Figure Legend” sections after which the media was aspirated and cells were washed 1X in ice cold PBS, pH 7.4. 200 μL of cytosolic buffer (provided in the kit, diluted 1:5 in ddH₂O, with added protease inhibitor cocktail and 1mM DTT as per the manufacturer’s recommendations) was added to the cells and allowed to incubate on ice for 20 min. Cells were scraped and harvested, then homogenized with 40 passes of a dounce homogenizer. Samples were spun down at 720 rcf for 10 min at 4°C. The supernatant from each sample was transferred to a new tube labeled mitochondrial fraction and spun at 10,000 rcf for 30 min at 4°C. The supernatant was then transferred to a new tube labeled cytosolic fraction and the pellet in the mitochondrial fraction tube was resuspended in 150 μL mitochondrial buffer (provided in the kit, with added protease inhibitor cocktail and 1mM DTT as per the manufacturer’s recommendations). Samples were measured for glutathione content as described below.

2.3.8 Glutathione Assay

Total Glutathione (GSH+GSSG) was measured using an assay kit (DTNB) from Oxford Biomedical, following the manufacturer’s protocol. All glutathione measurements were normalized to protein concentration.
2.3.9 MDA Assay

MDA was measured using an MDA assay kit from OxisResearch, following the manufacturer’s protocol. All MDA measurements were normalized to protein concentration.

2.3.10 Immunoblot analysis

Immunoblot analysis was completed as previously described in (Zimmermann et al., 2007).

2.3.11 GSH Transport Assay

GSH transport into isolated mitochondria was measured as previously published (Zimmermann et al., 2007). 20 μL of rat brain mitochondria were added to 230 μL of GSH transport buffer (5 mM HEPES, pH 7.2, 220 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 0.1% BSA (fatty acid-free), 5 mM succinate, and 1 mM potassium phosphate) at room temperature. The isolated mitochondria were then preincubated for 20 min with either 0.5 μL Me₂SO (vehicle, Con), 20 μM HA14-1 (HA14), or 2 μM recombinant Bim (Bim). A trace amount (0.5 μCi) of radiolabeled [³H]GSH in buffer containing unlabeled (cold) GSH at a final concentration of 50 μM was then added to the isolated mitochondria, vortexed and incubated for 15 sec at room temperature. 1 mL volume of ice-cold transport buffer was then added to each tube, and the samples were pelleted and washed 2X with 500 μL ice-cold buffer, and then 100 μL of 1N NaOH was added to dissolve the final pellets. [³H]GSH uptake into the isolated mitochondria was counted by liquid scintillation. The data were calculated as percentages of [³H]GSH uptake relative to Con.
2.3.12 Isolation and treatment of rat brain mitochondria

Mitochondria were isolated as previously described in (Zimmermann et al., 2007). Mitochondria were treated as follows; for 4 h GSH-MEE Co-incubation, mitochondria were incubated with 2 mM GSH-MEE in combination with either vehicle (Con), 20 μM HA14-1, or 10 μM rotenone for 4 h 37°C, 300 rpm. For the 2 h GSH-MEE pre-load/2 h wash out, mitochondria were pre-incubated with 2 mM GSH-MEE for 2 h, 37°C, 300 rpm, washed 2X with mitochondrial isolation buffer, and then incubated with either vehicle (Con), 20 μM HA14-1, or 10 μM rotenone for 2 h, 37°C, 300 rpm. All described treatments were completed in mitochondrial isolation buffer; 0.64 M sucrose, 2mM EDTA, and 20mM Tris-HCl, pH 7.4. The isolated mitochondria were then washed 3X in mitochondrial isolation buffer and assayed for glutathione content (described above).

2.3.13 Immunoprecipitation

CHO cells transfected with Bcl-2 and V5-OGC (5 μg each) were treated with either vehicle or 2 mM GSH-MEE for 4 h, 37°C, 10% CO₂ and lysed with 0.1% Triton X-100/Wahl buffer as described previously (Zimmermann et al., 2007), containing either 1 mM DTT or 2 mM GSH-MEE. Lysates were then immunoprecipitated using a monoclonal V5 antibody in 0.1% Triton X-100/Wahl buffer containing either 1 mM DTT or 2 mM GSH-MEE overnight, 4°C, mixing by inversion. Next, 50 μL or protein A/G agarose beads were incubated with the samples for 4 h, 4°C, mixing by inversion. Immune complexes were washed 3X and resolved by SDS-PAGE as previously described (Zimmermann et al., 2007). Whole cell lysates contained 200 μg of protein. Immunoblot analysis was completed for Bcl-2 (using Mouse TrueBlot® ULTRA Anti-
Mouse Ig HRP secondary antibody to eliminate the light chain) and V5. Primary CGNs were pre-incubated with 20 μM HA14-1 or vehicle (0.4%DMSO) for 2 h, 37°C, 10% CO₂ and samples were cross-linked with 500 μM DSS for 30 min, 37°C, 10% CO₂. CGNs were lysed in 1% Triton X-100/Wahl buffer with 50 mM glycine (to quench the cross-link), after which lysates were immunoprecipitated using a monoclonal GSH antibody and Protein A/G agarose beads as described above, and subjected to SDS-PAGE as described previously (Zimmermann et al., 2007). Recombinant immunoprecipitation experiments were performed using 50 ng of both recombinant Bcl-2 and recombinant GST-OGC, incubated in 0.1% Triton/Wahl buffer with 10 μM GSH overnight in the presence of either a monoclonal Bcl-2 antibody or a monoclonal Slc25a11 (OGC) antibody. Samples were immunoprecipitated as described above and subjected to SDS-PAGE as described previously (Zimmermann et al., 2007). Total samples contained 50 ng of both Bcl-2 and GST-OGC recombinant proteins.

2.4 Results

2.4.1 The BH3 mimetic, HA14-1, induces MOS and apoptosis of CGNs

Consistent with our previous observations (Zimmermann et al., 2005; Zimmermann et al., 2007), incubation of CGNs with the BH3 mimetic, HA14-1, induced an increase in active caspase-3, degradation of the microtubule network, and nuclear fragmentation indicative of apoptosis (Figure 2.1.A). Exposure of CGNs to HA14-1 significantly increased the concentration of malondialdehyde (MDA) in mitochondrial fractions, demonstrating an induction of MOS (Figure 2.1.B).
Figure 2.1 The BH3 mimetic, HA14-1, induces mitochondrial oxidative stress and apoptosis of CGNs

A. CGNs were treated for 24 h with either HA14-1 (HA14; 15 μM) or vehicle, control (Con, 0.3% DMSO). Top panels: Ftc, tubulin; DAPI, nuclei; Cy3, active caspase-3. Arrows indicate apoptotic cells with either condensed/fragmented nuclei, or immunoreactivity for active caspase-3. Bottom panels: decolorized DAPI images to show nuclear morphology.

B. CGNs were treated with either vehicle (Con) or HA14-1 (HA14; 20 μM) for 4 h and then fractionated into cytosolic and mitochondrial fractions. Mitochondrial fractions were then measured for MDA concentration, or lipid peroxidation levels. Data are shown as μ moles of MDA present per μg of protein and analyzed using a Student’s T-test, ***p<0.001, n=4.
2.4.2 HA14-1 selectively depletes the mitochondrial glutathione pool in CGNs

We have previously shown that CGN apoptosis induced by HA14-1 is prevented by glutathione (Zimmermann et al., 2005; Zimmermann et al., 2007). Therefore, we measured mitochondrial and cytosolic pools of total glutathione in CGNs incubated in the absence and presence of HA14-1. CGNs treated with HA14-1 for 4 h displayed selective depletion of total mitochondrial glutathione to approximately 50% of the control level (Figure 2.2.A). In contrast, CGNs incubated with ethacrynic acid, a compound that forms nonfunctional adducts with GSH, showed significant depletion of both the mitochondrial and cytosolic pools of glutathione (Figure 2.2.A). Cox-IV blots were performed to demonstrate pure fractionation of mitochondria and cytoplasm (Figure 2.2.B). The fraction of total cellular glutathione made up by mitochondrial (~10%) versus cytosolic (~90%) pools were consistent with previous studies (Figure 2.2.C). In addition, control levels of total mitochondrial glutathione were an average of 6.78 +/- 0.33 nmoles/mg of protein (n=4) and these results are consistent with previous studies (Ravindranath et al., 1989; Sagara et al., 1993; Huang and Philbert, 1995).
Figure 2.2 HA14-1 selectively depletes mitochondrial glutathione in CGNs

A. CGNs treated with either vehicle (Con), HA14-1 (HA14; 20 μM), or ethacrynic acid (EA; 100 μM) for 4 h and then separated into mitochondrial and cytosolic fractions. Total glutathione content of each fraction was measured using a DTNB colorimetric assay. Data are shown as percent of control glutathione (GSH+GSSG) and analyzed using one-way ANOVA with a post-hoc Tukey’s analysis. *p<0.05, **p<0.01 vs Con, n=4. Control levels of total mitochondrial glutathione were an average of 6.78 +/- 0.33 nmoles per mg of protein.

B. Immunoblot shown is for Cox-IV to demonstrate the purity of the mitochondrial and cytosolic fractions in (A). C. Graph showing the representative pools of cellular glutathione between mitochondrial and cytosolic fractions, n=4.
2.4.3 Bcl-2 co-immunoprecipitates with GSH following chemical cross-linking in CGNs; an interaction antagonized by the BH3 mimetic, HA14-1

We have previously shown that recombinant Bcl-2 directly interacts with GSH in vitro (Zimmermann et al., 2007). To determine if Bcl-2 interacts with GSH in intact cells we utilized a chemical cross-linking approach and measured the co-immunoprecipitation of Bcl-2 with GSH from CGNs. Cultured CGNs were cross-linked using DSS (disuccinimidyl suberate; a cell permeable, homobifunctional cross-linker possessing two amine-reactive N-hydroxysuccinimide esters), and upon quenching the cross-link with excess glycine, Bcl-2 was co-immunoprecipitated with GSH using a GSH antibody (Figure 2.3). In CGNs pre-incubated with DMSO vehicle and then cross-linked and immunoprecipitated with a GSH antibody, both monomeric and dimeric forms of Bcl-2 were co-immunoprecipitated with GSH. However, pre-treatment of the CGNs, prior to cross-linking, with HA14-1 significantly decreased the amount of both the monomeric and dimeric forms of Bcl-2 co-immunoprecipitated with GSH. These data strongly suggest that Bcl-2 and GSH interact in intact CGNs, and this interaction can be antagonized by the BH3 mimetic, HA14-1. Furthermore, we completed similar experiments in isolated rat brain mitochondria and found that HA14-1 also antagonized the interaction between Bcl-2 and GSH (data not shown). These findings are consistent with our previously published data in which we showed that recombinant Bcl-2 and GSH interact in a direct manner. Moreover, this interaction shows specificity between Bcl-2 and GSH, since other Bcl-2 family proteins, such as Bcl-xL and Bcl-w, did not interact directly with GSH (Zimmermann et al., 2007).
**Figure 2.3** *Bcl-2 co-immunoprecipitates with GSH following chemical cross-linking in CGNs, an interaction antagonized by the BH3 mimetic HA14-1* CGNs were pre-incubated with either vehicle (0.4% DMSO) or 20 μM HA14-1 for 2 h, after which they were cross-linked (x-link) with DSS, which was quenched upon lysing (see materials and methods). Lysates were immunoprecipitated (IP) with a monoclonal antibody to GSH. Immune complexes were resolved by SDS-PAGE and immunoblotted (IB) for Bcl-2. Mono=monomeric, dimer=dimeric, WCL=whole cell lysate.
2.4.4 HA14-1 depletes glutathione from isolated mitochondria. HA14-1 and Bim inhibit mitochondrial GSH transport in vitro

We examined the effects of HA14-1 on glutathione pools in isolated rat brain mitochondria. Incubation of isolated mitochondria with HA14-1 induced a significant depletion of total glutathione (GSH+GSSG) when mitochondria were co-incubated in the presence of GSH-MEE (Figure 2.4.A, n=3, **p<0.01 vs. Con) for 4h. HA14-1 has previously been shown to have some uncoupling and inhibitory effects on mitochondrial respiration at high concentrations (Milanesi et al., 2006). To exclude the possibility that HA14-1 (used at 20 μM) was depleting glutathione in isolated rat brain mitochondria by inhibiting mitochondrial respiration, and in turn inducing oxidative stress, we compared its effects to those of the complex I inhibitor, rotenone, which we used at a concentration significantly higher than its previously shown IC$_{50}$ value for complex I (Chen et al., 2006; Janssen et al., 2007). While HA14-1 (20 μM) significantly depleted total mitochondrial glutathione content, rotenone (10 μM) had no such effect (Figure 2.4.A). In another series of experiments, we pre-loaded isolated mitochondria with 2 mM GSH-MEE for 2 h and then washed the mitochondria prior to adding HA14-1 or rotenone for an additional 2 h. Following the 2 h pre-incubation with GSH-MEE, mitochondrial glutathione content was increased by approximately 250% over non-loaded controls (data not shown). Incubation with HA14-1 induced a marked reduction of total glutathione in the pre-loaded mitochondria, suggesting that the Bcl-2 inhibitor actually stimulated glutathione efflux (Figure 2.4.B, n=6, *p<0.05 vs. Con). In contrast, incubation with rotenone had no effect
on mitochondria pre-loaded with GSH-MEE. We compared the amount of total glutathione in freshly isolated mitochondria versus the Con samples in the treatment paradigms of Figures 2.4.A and 2.4.B; no differences in the levels of total glutathione were observed (an average of 17.84 +/- 1.69 nmoles/mg, n=3, data not shown). Finally, we assessed the effects of HA14-1 or recombinant Bim on the transport of [³H]GSH into isolated mitochondria. Both Bcl-2 inhibitors, HA14-1 (20μM) or Bim (2μM), significantly inhibited GSH uptake into mitochondria (Figure 2.4.C, #p<0.01 vs Con and *p<0.05 vs Con, n=3). These data show that HA14-1 and Bim suppress mitochondrial GSH transport and that HA14-1 is capable of inducing efflux of glutathione from isolated mitochondria.
Figure 2.4 HA14-1 depletes glutathione from isolated rat brain mitochondria, in addition HA14-1 and Bim inhibit mitochondrial GSH transport A. Isolated rat brain mitochondria were incubated in mitochondrial buffer with a combination of 2 mM GSH-MEE and either vehicle (Con), HA14-1 (20 μM), or rotenone (10 μM) for 4 h. Samples were then washed 3X in mitochondrial buffer and assayed for glutathione (GSH+GSSG) content. Data are shown as percent of control glutathione (GSH+GSSG) and analyzed using a one-way ANOVA with a post-hoc Tukey’s analysis. ** p<0.01 vs Con, n=3. B. Isolated rat brain mitochondria were incubated in mitochondrial buffer with GSH-MEE (2 mM) for 2 h at 37°C, washed 2X, and then incubated in mitochondrial buffer with either Con, HA14-1 (HA14; 20 μM), or rotenone (10 μM) for 2h at 37°C. After which samples were washed 3X with mitochondrial buffer and assayed for glutathione (GSH+GSSG) content using a DTNB colorimetric assay, data are normalized to protein concentration. Two controls are shown because some of the experiments performed with HA14-1 were carried out separately from several of the experiments performed with rotenone. Data are represented as percent of the respective control glutathione (GSH/GSSG) and analyzed using a Student’s T-test. *p<0.05 vs Con, n=6. Control total mitochondrial glutathione levels for the isolated rat brain mitochondria experiments were an average of 17.84 +/− 1.69 nmoles/mg of protein. C. Isolated rat brain mitochondria were incubated with either vehicle (Con; 0.4%DMSO), HA14-1 (HA14; 20 μM) or Bim (2 μM) for 20 min and then 0.5μ Ci of [3H]GSH for 15 sec at room temperature. [3H]GSH was measured as described under Materials and Methods. Data are represented as percent of Con (CPM values) and analyzed using a one-way ANOVA with post-hoc Tukey’s analysis. **p<0.01 vs Con, *p<0.05 vs Con, n=3 experiments completed in triplicate. I acknowledge Kristin Marquardt for assistance in generating the data for this figure.
2.4.5 Bcl-2 co-immunoprecipitates with OGC and this interaction is enhanced by GSH-MEE

HA14-1, a BH3 mimic that binds Bcl-2 within the hydrophobic groove, selectively depletes mitochondrial glutathione and antagonizes the interaction between Bcl-2 and GSH in CGNs, and inhibits GSH uptake/stimulates glutathione efflux in isolated brain mitochondria. These data suggest a possible role for Bcl-2 in regulating mitochondrial glutathione transport. It has been previously shown that the IMM anion transporters OGC and DIC are responsible for glutathione transport across the inner mitochondrial membrane into the matrix in liver and kidney (Chen and Lash, 1998; Chen et al., 2000). Therefore, we next examined the potential of Bcl-2 to interact with the OGC in a transient co-transfection system. CHO cells overexpressing Bcl-2 and a V5-tagged OGC were lysed in 0.1% Triton X-100 with 1 mM DTT, and immunoprecipitated with or without a V5 antibody. Bcl-2 co-immunoprecipitated with V5-OGC upon addition of the V5 antibody but did not precipitate in the presence of protein A/G agarose beads alone (Figure 2.5.A). Next, we determined the effects of GSH-MEE on the interaction of Bcl-2 with OGC. CHO cells overexpressing Bcl-2 and V5-OGC were treated with 2 mM GSH-MEE for 4 h, and cells were lysed and immunoprecipitated in buffer also containing 2 mM GSH-MEE. In control conditions, CHO cells overexpressing Bcl-2 and V5-OGC were untreated, lysed, and immunoprecipitated in buffer containing 1 mM DTT. The relative amount of Bcl-2 co-immunoprecipitated with V5-OGC was appreciably increased in the presence of GSH-MEE versus DTT (Figure 2.5.B). Densitometric analysis using normalization of the Bcl-2 band intensity to the V5-OGC band intensity
revealed a significant increase (approximately five-fold) in Bcl-2 co-immunoprecipitation with V5-OGC in the presence of GSH-MEE versus DTT (Figure 2.5.C, n=9, p<0.05 vs. DTT). GSH-MEE enhanced the interaction between Bcl-2 and OGC irrespective of the time in which it was added into the experiment, such as treating the cells only or simply adding GSH-MEE to the immunoprecipitation buffer (data not shown). Moreover, in a cell free system, recombinant Bcl-2 also interacted directly with recombinant GST-tagged OGC in the presence of GSH (Figure 2.5.D). Both recombinant Bcl-2 and recombinant GST-OGC co-immunoprecipitated with a Bcl-2 antibody and a Slc25a11 (OGC) antibody (Figure 2.5.D). Finally, upon the addition of ethacrynic acid to the immunoprecipitation buffer (which already contained GSH therefore ethacrynic acid was used to deplete the GSH), the interaction between recombinant OGC and recombinant Bcl-2 was antagonized (Figure 2.5.E). These results indicate that Bcl-2 is an interacting partner for OGC and this novel protein-protein interaction is enhanced by GSH.
Figure 2.5 Bcl-2 co-immunoprecipitates with V5-OGC from co-transfected CHO cells, GSH-MEE enhances the interaction

A. CHO cells were co-transfected with Bcl-2 and V5-OGC (5μg each) lysed in Wahl buffer containing 0.1% TritonX-100 and 1 mM DTT, and immunoprecipitated (IP) with a monoclonal V5 antibody. Immune complexes were then resolved by SDS-PAGE, immunoblotted for V5 and Bcl-2. WCL= Whole cell lysate, IP = immunoprecipitate, −V5 antibody=Protein A/G agarose alone was used in the IP.

B. Same as in (A), except at 24 h post-transfection cells were treated with 2 mM GSH-MEE for 4 h (and GSH-MEE was added to the IP buffer). Alternatively, 1 mM DTT was added to the IP buffer.

C. Densitometry comparing pixel density of co-immunoprecipitated Bcl-2 with V5-OGC in the presence of either 1 mM DTT (Con) or 2 mM GSH-MEE. Data are normalized to the amount of V5 immunoprecipitated and are expressed as a percent of Bcl-2 immunoprecipitated in the presence of DTT (Con). Data was analyzed using a Student’s T-test, *p<0.05, n=9.

D. Recombinant Bcl-2 and recombinant GST-OGC were co-immunoprecipitated with either anti-Bcl-2 (Bcl-2 IP) or anti-Slc25a11 (OGC IP) in 0.1% Triton X-100/Wahl buffer with 11.1 μM GSH. Immune complexes were resolved by SDS-PAGE, immunoblotted for Bcl-2 and Slc25a11 (OGC).

E. Recombinant Bcl-2 and recombinant GST-OGC were co-immunoprecipitated with anti-Bcl-2 in either 0.1% Triton X-100/Wahl buffer with 10 μM GSH (−EA) or 0.1% Triton X-100/Wahl buffer with 10 μM GSH and 100 μM ethacrynic acid (+EA). Immune complexes were resolved by SDS-PAGE, immunoblotted for Bcl-2 and Slc25a11 (OGC).
2.4.6 Co-expression of Bcl-2 and OGC increases mitochondrial glutathione

We next examined the effects of Bcl-2 and OGC expression on mitochondrial glutathione content. Here, we utilized CHO cells expressing DsRed-2 (Con), Bcl-2, V5-OGC, or a combination of Bcl-2 and V5-OGC. At 24 h post-transfection, cells were lysed and mitochondrial and cytosolic fractions were obtained by differential centrifugation and measured for total glutathione content. Control, Bcl-2, and OGC expressing CHO cells showed similar mitochondrial glutathione content (Figure 2.6.A). However, with the co-expression of both Bcl-2 and OGC, total mitochondrial glutathione was significantly increased compared to Bcl-2 expression alone (Figure 2.6.A, n=3, *p<0.05 vs. Bcl-2/OGC). Immunoblot analysis confirmed the expression of Bcl-2 and OGC in the appropriate mitochondrial fractions (Figure 2.6.B), and a Cox-IV blot demonstrated the relative purity of the mitochondrial fractions (Figure 2.6.C). Control levels of total mitochondrial glutathione were an average of 12.69 +/- 1.81 nmoles/mg of protein. These data suggest that Bcl-2 and OGC work in a concerted manner to increase the mitochondrial glutathione pool.
Figure 2.6 Co-transfection with Bcl-2 and V5-OGC significantly increases mitochondrial glutathione content

A. CHO cells were transfected with control (DsRed), Bcl-2, V5-OGC, or a combination of Bcl-2 and V5-OGC (5μg each). After 24 h, cells were fractionated and mitochondrial glutathione (GSH+GSSG) content was measured using DTNB, and normalized to protein concentration. Data are expressed as percent of control glutathione and analyzed using a one-way ANOVA with a post-hoc Tukey’s analysis *p<0.05 vs Bcl-2/OGC, n=3. Control levels of total mitochondrial GSH were at an average of 12.69 +/- 1.81 nmoles/mg of protein.

B. Immunoblots of mitochondrial fractions indicating expression of Bcl-2, V5-OGC, or a combination of both in transfected CHO cells.

C. Immunoblot for Cox-IV indicating purity of mitochondrial and cytosolic fractions.
2.4.7 Bcl-2 protection from hydrogen peroxide toxicity is dependent on the function of OGC

Finally, we examined the consequences of increasing the mitochondrial glutathione pool via co-expression of Bcl-2 and OGC in transiently transfected CHO cells. In agreement with many previous studies (Hockenbery et al., 1993; Satoh et al., 1996; Papdopoulous et al., 1998), overexpression of Bcl-2 alone significantly protected CHO cells from hydrogen peroxide-induced apoptosis as assessed by measuring the percentage of adherent cells expressing active caspase-3 (Figure 2.7.A, n=4, *p<0.05 vs Con+H_2O_2, **p<0.01 vs Con+H_2O_2). In a similar manner, expression of either OGC alone or OGC in combination with Bcl-2 also significantly protected cells from hydrogen peroxide. Interestingly, co-incubation of CHO cells with the OGC inhibitor, phenylsuccinate (PhS), at a concentration (500 μM) which was titrated down to a maximally non-cytotoxic level, did not significantly enhance the active caspase-3 staining either on its own or in combination with the hydrogen peroxide exposure (Figure 2.7.B). Overexpression of OGC, in either the absence or presence of Bcl-2, maintained its protective effect against hydrogen peroxide even in the presence of this low dose of PhS (Figure 2.7.C, n=4, *p<0.05 vs Con+PhS and H_2O_2, #p<0.01 vs Bcl-2+PhS and H_2O_2). These results are consistent with the low dose of PhS being sufficient to only inhibit the transport capabilities of the endogenous OGC within CHO cells. In marked contrast to the effects observed with OGC, Bcl-2 appeared to lose its protective effect against hydrogen peroxide when the endogenous levels of OGC within CHO cells were simultaneously inhibited by PhS. This point is demonstrated by the findings that Bcl-2
alone was ineffective at attenuating caspase-3 activation in CHO cells treated concomitantly with PhS and hydrogen peroxide (Figure 2.7.C). These results suggest that Bcl-2 protects cells from hydrogen peroxide-induced apoptosis via an OGC-dependent mechanism (i.e., via an increase in mitochondrial glutathione transport resulting in an enhancement of the mitochondrial glutathione pool).
Figure 2.7 Bcl-2 protection against $H_2O_2$-induced apoptosis is OGC-dependent

A. CHO cells transfected with DsRed, Bcl-2, V5-OGC, or a combination of Bcl-2 and V5-OGC were treated with 100 μM $H_2O_2$ for 24 h and stained for active caspase-3. Data are represented as mean +/- SEM. Data were analyzed using a one-way ANOVA with a post-hoc Tukey’s analysis. *p<0.05 vs Con+$H_2O_2$, **p<0.01 vs Con+$H_2O_2$, *** p<0.001 vs Con+$H_2O_2$, and #p<0.001 vs Con, n=4.

B. CHO cells transfected as in (A) and treated with either vehicle, 100 μM $H_2O_2$ for 24 h alone or in combination with 500 μM phenylsuccinate (PhS), or 500 μM phenylsuccinate (PhS) alone for 24 h and stained for active caspase-3. NS=not significant, data are represented as mean +/- SEM and analyzed using a Student’s T-test.

C. CHO cells transfected as in (A), treated with a combination of 100 μM $H_2O_2$ and 500 μM phenylsuccinate (PhS) for 24 h and stained for active caspase-3. Data are represented as mean +/- SEM. Data were analyzed using a one-way ANOVA with a post-hoc Tukey’s analysis. *p<0.05 vs Con+PhS and $H_2O_2$ and **p<0.01 vs Con+PhS and $H_2O_2$, n=4.
2.5 Discussion

Bcl-2 is a key sentinel of the mitochondria that acts to suppress the intrinsic apoptotic cascade via its inhibitory interactions with pro-apoptotic family members (Chipuk et al., 2008). Beyond its classical anti-apoptotic role, Bcl-2 is also known to have a critical antioxidant-like function (Hockenbery et al., 1993). This antioxidant-like property has been linked experimentally to the regulation of cellular glutathione content (reviewed in Voehringer, 1999). For example, overexpression of Bcl-2 increases cellular glutathione via an enhancement of glutathione synthesis and a diminution of cellular glutathione efflux (Ellerby et al., 1996, Meredith et al., 1998; Jang et al., 2004).

Accordingly, overexpression of Bcl-2 protects cells from oxidative stress induced by glutathione depleting agents (Kane et al., 1993; Merad-Saïdoune et al., 1999; Rimpler et al., 1999). Furthermore, the protective effects of Bcl-2 against oxidative damage are significantly diminished by depriving cells of glutathione precursors, for instance through incubation in cysteine/methionine-deficient media (Mirkovic et al., 1997). Finally, Bcl-2 null mice show altered antioxidant enzyme activities in brain, such as decreased glutathione peroxidase activity, and demonstrate increased sensitivity to neural oxidative injury (Hochman et al., 1998). Many studies have provided evidence of a connection between cellular glutathione status and the antioxidant-like action of Bcl-2. However, the specific molecular mechanism(s) underlying this relationship between Bcl-2 and glutathione has not yet been elucidated.
Previously, a novel interaction between recombinant Bcl-2 and GSH in a cell-free assay was demonstrated (Zimmermann et al., 2007). We showed that two dissimilarly structured BH3 mimetics predicted to bind in the hydrophobic surface groove (i.e., the BH3 groove) of Bcl-2, HA14-1 and compound 6, each antagonized this novel Bcl-2/GSH interaction, as did recombinant Bim (Zimmermann et al., 2007). From these previous data, we concluded that Bcl-2 is a de facto GSH-binding protein and we hypothesized that this newly discovered property of Bcl-2 might play a central role in its antioxidant-like function at mitochondria. Consistent with our hypothesis, we report here that HA14-1 induces a specific depletion of the mitochondrial glutathione pool in CGNs and this BH3 mimetic inhibits the Bcl-2/GSH interaction in intact CGNs. In addition, HA14-1 and Bim inhibit the mitochondrial uptake of [³H]GSH and HA14-1 induces glutathione efflux in isolated rat brain mitochondria. Importantly none of these results are observed in isolated mitochondria incubated with the complex I inhibitor, rotenone, demonstrating that the effects of HA14-1 on mitochondrial glutathione transport are independent of any inhibitory actions on the mitochondrial respiratory machinery.

To further establish a link between Bcl-2 function and mitochondrial glutathione transport, we used a transient co-transfection system in CHO cells to demonstrate a novel interaction between Bcl-2 and the IMM glutathione transporter, OGC. Significantly, this unique protein-protein interaction is markedly enhanced by GSH-MEE. We also show the interaction between OGC and Bcl-2 is direct through the use of recombinant proteins in an immunoprecipitation and this interaction is dependent on glutathione because the
addition of ethacrynic acid (which binds to GSH and forms non-functional adducts) significantly abolished the ability of OGC and Bcl-2 to bind. Additional experiments using co-transfected CHO cells reveal that Bcl-2 and OGC work in conjunction to significantly increase the mitochondrial glutathione pool. Finally, we demonstrate that the ability of Bcl-2 to protect cells from oxidative stress is largely dependent on an intact transporter function of OGC because phenylsuccinate (an OGC inhibitor) abolishes the protective effect of Bcl-2 against hydrogen peroxide-induced apoptosis in CHO cells.

Based on our current and previously published data, we propose the following model to explain how Bcl-2 acts as a central regulator of the mitochondrial glutathione pool. Under normal circumstances, OGC functions as an IMM glutathione transporter that helps to maintain an adequate mitochondrial glutathione pool. We suggest that Bcl-2 localized to the outer mitochondrial membrane acts as a “sensor” of cytoplasmic glutathione content, and upon GSH-binding, a conformational change is induced within Bcl-2 that increases its affinity for OGC. The Bcl-2/OGC interaction in turn, enhances the mitochondrial glutathione transporter function of OGC to increase the mitochondrial glutathione pool. In this manner, an increase in cytoplasmic glutathione content, which may occur via either enhanced synthesis or reduced efflux (Meredith et al., 1998; Jang et al., 2004), may be transduced into the mitochondria to induce a corresponding elevation in mitochondrial glutathione content. However, when HA14-1 or Bim binds to Bcl-2 within the BH3 groove, this antagonizes (either directly or allosterically) the interaction between GSH and Bcl-2, and as a consequence lowers the affinity of Bcl-2 for OGC.
Thus, BH3 mimetics and BH3-only proteins inhibit the capacity of Bcl-2 to regulate mitochondrial glutathione transport through OGC, leading to a selective depletion of mitochondrial glutathione, an increase in MOS, and activation of the intrinsic apoptotic cascade.

Our proposed model is supported by several previously published observations. First, in addition to our results showing that the BH3 mimic, HA14-1, depletes mitochondrial glutathione in CGNs, other BH3 mimetics have been shown to have similar effects in different cell types. The highly selective Bcl-2 inhibitor, ABT-737, has been shown to deplete cellular glutathione in Jurkat cells and HeLa cells, although mitochondrial glutathione content was not explicitly measured in this study (Howard et al., 2009). These findings, along with our data showing that Bim directly inhibits GSH uptake into isolated mitochondria, strongly suggest that inhibition of Bcl-2 is a common mechanism of depleting mitochondrial glutathione. Second, there is a precedent for Bcl-2 to interact with and modulate the activity of IMM transporters. For instance, Bcl-2 has been shown to enhance the ADP/ATP exchange activity of the IMM adenine nucleotide translocase in proteoliposomes, isolated mitochondria, and intact cells (Belzacq et al., 2003). Interestingly, Bax displaces Bcl-2 from the translocase and acts to inhibit the ADP/ATP exchange activity. Third, a previous study demonstrated a positive correlation between an accumulation of Bcl-2 in the nuclear membrane and a redistribution of glutathione into the nucleus, adding further support to a central role for Bcl-2 in the regulation of glutathione transport that is not restricted to mitochondria (Voehringer et
al., 1998). Finally, the ability of BH3 mimetics and BH3-only proteins (e.g., Bim) to disrupt the Bcl-2/GSH interaction is entirely consistent with the pro-oxidant state induced at mitochondria by pro-apoptotic Bcl-2 family members (Kirkland et al., 2002; Starkov et al., 2002; Ding et al., 2004).

Collectively, the results described above suggest that Bcl-2 plays a central role in the regulation of mitochondrial glutathione transport and maintenance of the mitochondrial glutathione pool. Moreover, this key antioxidant-like function of Bcl-2 likely involves direct GSH-binding and a GSH-stimulated interaction with the IMM GSH transporter, OGC. Gallo et al. found a similar interaction between the C. elegans homolog of OGC (MISC-1) and the Bcl-2 homolog (Ced-9), further providing evidence for an interaction between Bcl-2 and OGC (Gallo et al., 2011).

Given the substantial roles of glutathione depletion and MOS in neurodegeneration, one could postulate how negatively impacting this novel function of Bcl-2 might contribute to the pathophysiology of various neurodegenerative diseases. For example, GSH depletion in the spinal cord of mice harboring a familial ALS mutation (G93A) in the SOD1 gene has been shown to correlate with motor neuron degeneration during disease progression (Chi et al., 2007). In transgenic mice and rats, as well as human familial ALS patients, various mutant forms of SOD1 are apparently recruited to spinal cord mitochondria (Liu et al., 2004). In general, these mutant forms of SOD1 are observed as misfolded aggregates that have been localized to essentially every compartment of the mitochondria; however, mutant SOD1 has also been shown to
aggregate with Bcl-2 at mitochondria (Pasinelli et al., 2004). Most significantly, this interaction of mutant SOD1 with Bcl-2 has been shown to induce a conformational change in Bcl-2 that exposes its BH3 domain and converts Bcl-2 into a “toxic” protein at mitochondria (Pedrini et al., 2010). Although the authors of this elegant study support a direct toxic function of the conformationally altered form of Bcl-2 at mitochondria, it is tempting to speculate that the interactions of Bcl-2 with mutant SOD1 may also interfere with the novel role that Bcl-2 plays in regulating mitochondrial glutathione transport. For instance, if only a fraction of Bcl-2 is actually altered in conformation by its interaction with mutant SOD1, then this “toxic” form of Bcl-2 may in fact act like a BH3-only protein and disrupt the ability of adjacent unaltered Bcl-2 molecules to bind GSH and stimulate OGC-dependent glutathione transport into the mitochondria. In fact, this model is consistent with findings in NSC34 motor neuronal cells stably expressing G93A mutant SOD1. These cells demonstrate a significant depletion of the mitochondrial glutathione pool with no effect on cytosolic glutathione content (Muyderman et al., 2009). Moreover, these mutant SOD1-expressing cells demonstrate a more pronounced depletion of mitochondrial glutathione and display an enhanced sensitivity to apoptosis in response to ethacrynic acid when compared to non-transfected or wild type SOD1-expressing NSC34 cells (Rizzardini et al., 2005; Muyderman et al., 2009). Thus, motor neuron toxicity induced by mutant SOD1 likely involves the interaction of this protein with Bcl-2 in the outer mitochondrial membrane. The precise consequences of this interaction, if any, on the capacity of Bcl-2 to regulate mitochondrial glutathione transport requires further study.
In conclusion, we identify the regulation of mitochondrial glutathione transport and maintenance of the mitochondrial glutathione pool as essential and novel functions of Bcl-2. We establish Bcl-2 as a direct GSH-binding protein and a novel interacting partner for the IMM glutathione transporter, OGC. Either BH3 mimetics or BH3-only proteins (e.g., Bim) interfere with the Bcl-2/GSH interaction and result in inhibition of mitochondrial glutathione transport. Bcl-2 and OGC appear to work in a concerted manner to significantly increase the mitochondrial glutathione pool. Accordingly, the ability of Bcl-2 to protect cells from oxidative stress is largely dependent on an intact transporter function of OGC. These findings suggest a molecular mechanism for the well-established antioxidant-like function of Bcl-2, particularly at the level of the mitochondria. Finally, this novel function of Bcl-2 may be a target of pathogenic proteins, such as mutant SOD1, which result in devastating neurodegenerative disorders like ALS.
CHAPTER THREE: MITOCHONDRIAL GLUTATHIONE TRANSPORT IS A KEY DETERMINANT OF NEURONAL SUSCEPTIBILITY TO OXIDATIVE AND NITROSATIVE STRESS

3.1 Abstract

Mitochondrial oxidative stress significantly contributes to the underlying pathology of several devastating neurodegenerative disorders. Mitochondria are highly sensitive to the damaging effects of reactive oxygen and nitrogen species; therefore, these organelles are equipped with a number of free radical scavenging systems. In particular, the mitochondrial glutathione (GSH) pool is a critical antioxidant reserve that is derived entirely from the larger cytosolic pool via facilitated transport. The mechanism of mitochondrial GSH transport has not been extensively studied in the brain. However, the dicarboxylate (DIC) and 2-oxoglutarate (OGC) carriers localized to the inner mitochondrial membrane have been established as GSH transporters in liver and kidney. Here, we investigated the role of these carriers in protecting neurons from oxidative and nitrosative stress. Immunoblot analysis of DIC and OGC in primary cultures of rat cerebellar granule neurons (CGNs) and cerebellar astrocytes showed differential expression of these carriers, with CGNs expressing only DIC and astrocytes expressing both DIC and OGC. Consistent with these findings, butylmalonate specifically reduced mitochondrial GSH in CGNs, whereas both butylmalonate and phenylsuccinate diminished mitochondrial GSH in astrocytes. Moreover, preincubation with butylmalonate but not phenylsuccinate significantly enhanced susceptibility of
CGNs to oxidative and nitrosative stressors. This increased vulnerability was largely prevented by incubation with cell-permeable GSH monoethylester but not malate. Finally, knockdown of DIC with adenoviral siRNA also rendered CGNs more susceptible to oxidative stress. These findings demonstrate that maintenance of the mitochondrial GSH pool via sustained mitochondrial GSH transport is essential to protect neurons from oxidative and nitrosative stress.

3.2 Introduction

Mitochondrial oxidative stress and mitochondrial dysfunction are convergence points in the underlying pathologies of several devastating neurodegenerative disorders (Lin and Beal, 2006). For instance, previous studies have provided significant evidence for increased mitochondrial oxidative damage and organelle dysfunction in Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS) (Murata et al., 2008; Arthur et al., 2009; Isobe et al., 2010). Overall, mitochondrial dysfunction leads to an increase in reactive oxygen species (ROS), which damage mitochondrial DNA, lipids, and proteins (Lin and Beal, 2006). Moreover, ROS induced alterations in mitochondrial membrane potential and permeability can trigger a positive feedback mechanism known as ROS-induced ROS release, which has the potential to propagate oxidative stress signals from mitochondria to mitochondria, thus exacerbating cellular injury (Zorov et al., 2006). These findings indicate that mitochondrial free radical scavenging systems, such as the essential antioxidant glutathione (GSH), are critical to protect neuronal cells from mitochondrial oxidative stress. GSH exists as a discrete mitochondrial pool and a much
larger cytosolic store. The mitochondrial GSH pool is considered to be an indispensable antioxidant reservoir. For example, selective depletion of the mitochondrial GSH pool within cerebellar granule neurons (CGNs) induced opening of the mitochondrial permeability transition pore, increased ROS production, and caused significant cell death, whereas depletion of the cytosolic GSH pool did not elicit these deleterious effects (Wüllner et al., 1999). In a similar manner, specific depletion of mitochondrial GSH in astrocytes rendered these glial cells more vulnerable to both oxidative and nitrosative stress (Muyderman et al., 2007). Based on these previous studies, mitochondrial GSH appears to be a critical antioxidant that supports both glial and neuronal cell viability.

Several factors indicate that GSH transport into mitochondria occurs via a facilitated process. These factors include a lack of the enzymes necessary for GSH synthesis within mitochondria, the negative potential of the inner mitochondrial membrane, the net negative charge of GSH at physiological pH, and the approximately equal concentrations of GSH in the cytosolic and mitochondrial compartments (Griffith and Meister, 1985; Lash, 2006). Previous studies have shown that two inner mitochondrial membrane anion transporters, the dicarboxylate carrier (DIC, \textit{Slc25a10}) and the 2-oxoglutarate carrier (OGC, \textit{Slc25a11}), are capable of transporting GSH into renal and hepatic mitochondria (Chen and Lash, 1998; Chen et al., 2000; Lash, 2006). Mechanisms of mitochondrial GSH transport in the brain have only recently been investigated. Using isolated mitochondria from whole brain, one study determined that mitochondrial GSH transport may depend on the function of the tricarboxylate carrier (Wadey et al., 2009). A second study showed that cortical neurons and astrocytes express both DIC and OGC. However,
in isolated mitochondria from whole cortical tissue, only the activity of DIC appeared to influence mitochondrial GSH content (Kamga et al., 2010). To date, the mechanism of mitochondrial GSH transport utilized by neurons specifically has not been examined. Furthermore, the effects of inhibiting either DIC- or OGC-dependent mitochondrial GSH transport on neuronal susceptibility to oxidative or nitrosative stress are unknown. Here, we demonstrate a key role for DIC-dependent mitochondrial GSH transport in the maintenance of the mitochondrial GSH pool and protection of CGNs from oxidative and nitrosative stress.

3.3 Materials and Methods

3.3.1 Materials

Butylmalonic acid, phenylsuccinic acid, primary antibody against β-tubulin, poly-L-lysine, GSH monoethylester, L-malate, and Hoechst stain were purchased from Sigma-Aldrich. HA14-1 was acquired from Alexis Biochemicals (Enzo Life Sciences, Plymouth Meeting, PA). The GSH assay kit was purchased from Oxford Biomedical (Rochester Hills, MI). Anti-MAP2 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The mitochondrial/cytosolic fractionation kit was from Biovision (Mountain View, CA). Antibody against Cox-IV was obtained from Cell Signaling (Beverly, MA). Primary rat astrocyte cultures were purchased from ScienCell (Carlsbad, CA). Anti-GFAP, anti-GAPDH, anti-DIC (Slc25a10), and anti-OGC (Slc25a11) antibodies were purchased from Abcam (Cambridge, MA). Sodium nitroprusside (SNP) was received from Calbiochem. Horseradish peroxidase conjugated secondary antibodies and reagents for enhanced chemiluminescence detection were purchased from Amersham Biosciences. The
ViraBind® adenovirus miniprep kit and QuickTiter™ adenovirus titer immunoassay kit were purchased from Cell Biolabs (San Diego, CA). Adenoviral siRNA1 and siRNA2 against DIC and the scrambled adenoviral control were constructed as described previously (Huypens et al., 2011). siRNA1 targets DIC from bp 119GCU UCG AAU GAC UGG AAU G, and siRNA2 targets DIC from bp 354GCA GAU UUG GUC AAU GUC A, whereas the scrambled siRNA sequence (GAG ACC CTA TCC GTG ATT A) has no known gene homology (Huypens et al., 2011).

3.3.2 CGN Cell Culture

CGNs were isolated and cultured from P7 Sprague-Dawley rat pups based on the methods described previously (Zimmermann et al., 2007). CGNs were maintained in basal modified Eagle’s medium, containing 10% heat inactivated fetal bovine serum, 25 mM potassium chloride, 2 mM L-glutamine and 100 units/ml/100 μg/ml penicillin/streptomycin at 37 °C in 10% CO₂.

3.3.3 Primary Rat Cerebellar Astrocyte Cell Culture

Primary rat cerebellar astrocyte cultures were grown on poly-L-lysine coated flasks in basal modified Eagle’s medium containing 10% heat-inactivated fetal bovine serum, 2mM-L-glutamine, and 100 units/ml/100 μg/ml penicillin/streptomycin. Cells were plated on poly-L-lysine-coated 6-well plates and grown to ~80% confluence at 37 °C in 10% CO₂, at which time cells were utilized for the described experiments.

3.3.4 Immunofluorescence Staining

CGNs and/or astrocytes were treated as described under “3.4 Results” and fixed with 4% paraformaldehyde. Cells stained for β-tubulin were blocked and permeabilized in 5%
BSA, 0.2% Triton X-100 in PBS for 1 h. Cells were then incubated with primary antibody overnight at 4 °C, at a dilution of 1:500 in 2% BSA, 0.2% Triton X-100 in PBS. Cells were then washed five times in PBS and incubated in secondary antibody for 1 h at room temperature at a dilution of 1:250 in 2% BSA, 0.2% Triton X-100 in PBS. Cells were then washed five times and placed in anti-quench solution. For cells with only nuclei stained, cells were fixed as above and incubated with Hoechst dye (8 μg/ml) in PBS for 1 h at room temperature. Cells were then washed five times with PBS and placed in antiquench. Images were captured using a Zeiss Axiovert-zoom inverted epifluorescence microscope.

3.3.5 Mitochondrial/Cytosolic Fractionation

Cells were treated as indicated under “3.4 Results” or in the figure legends, after which the medium was aspirated, and cells were washed once in ice-cold PBS, pH 7.4. 200 μl of cytosolic buffer (provided in the kit, diluted 1:5 in double-distilled H2O, with added protease inhibitor mixture and 1 mM DTT as per the manufacturer’s recommendations) was added to the cells and allowed to incubate on ice for 20 min. Cells were scraped and harvested and then homogenized with 40 passes of a Dounce homogenizer. Samples were spun down at 720 relative centrifugal force for 10 min at 4 °C. The supernatant from each sample was transferred to a new tube labeled “mitochondrial fraction” and spun at 10,000 relative centrifugal force for 30 min at 4 °C. The supernatant was then transferred to a new tube labeled “cytosolic fraction,” and the pellet in the mitochondrial fraction tube was resuspended in 150 μl of mitochondrial buffer (provided in the kit, with added protease inhibitor mixture and 1 mM DTT as per
the manufacturer’s recommendations. Samples were measured immediately for GSH content as described below.

3.3.6 GSH Assay

GSH was measured using an assay kit (DTNB) from Oxford Biomedical Research, following the manufacturer’s protocol. All GSH measurements were normalized to protein concentration. GSH concentrations were determined using a GSH standard in the DTNB assay and reported as nmol of GSH/mg of protein. The percentage of mitochondrial GSH and percentage of cytosolic GSH was calculated using the slope of the kinetic assay and the appropriate dilution factor.

3.3.7 Cell Lysis

CGNs were infected with the indicated adenoviral construct (shown under “3.4 Results”) as described below, after which CGNs were washed in 1 mL PBS over ice and incubated with 1% Triton X-100 in Wahl buffer (20 mM HEPES, pH 7.4, 50 mM NaCl, 1 mM EGTA, 5 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 100 μM sodium orthovanadate, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) for 10 min. Cells were harvested by scraping and spun down at 10,000 rpm for 2 min, and the cell supernatant was removed.

3.3.8 Immunoblot Analysis

Following mitochondrial/cytosolic fractionation or cell lysis, a BCA protein assay (Pierce) was used to determine protein concentration. Equal concentrations of protein were resolved by SDS-PAGE and immunoblotted as described previously (Zimmermann et al., 2007).
3.3.9 Purification and Titering of Adenoviral Constructs

Adenoviral constructs were amplified in a HEK 293 AD cell line by adding 10 μl of the respective adenovirus to 4 ml of medium for 1.5 h, after which 16 ml of medium was added to the flask, and cells were incubated at 5% CO2 and 37 °C for 48 h or until the beginning signs of cytotoxicity were observed. Cells were harvested, and the adenoviral constructs were purified as per the manufacturer’s recommendations in the ViraBind® adenovirus miniprep kit. Adenoviral constructs were titered as per the manufacturer’s recommendations in the QuickTiter™ adenovirus titer immunoassay kit.

3.3.10 Adenoviral Knockdown of DIC

CGNs were infected with adenoviral constructs at a multiplicity of infection of 100 for 48 h, after which experiments were completed.

3.3.11 Statistical Analysis

One-way analysis of variance tests followed by post hoc Tukey’s tests were performed to determine statistical significance between data sets. A p value of less than 0.05 was considered statistically significant. A minimum of three independent experiments were completed for each figure.

3.4 Results

3.4.1 Differential Expression of the Mitochondrial GSH Transporters, OGC and DIC, within CGNs and Cerebellar Astrocytes

To determine the specific cellular expression of the previously identified mitochondrial GSH transporters within the cerebellum, we employed primary rat CGN
and primary rat cerebellar astrocyte cultures (Fig. 3.1.A). We first completed immunoblots against DIC and OGC on mitochondrial fractions of these primary cultures. Here, we show that CGNs express DIC but no detectable OGC. In contrast, cerebellar astrocytes express both DIC and OGC (Fig. 3.1.B).
Figure 3.1 Differential expression of the mitochondrial GSH transporters, OGC and DIC, within CGNs and cerebellar astrocytes A. primary rat CGN cultures were fixed and stained for β-tubulin (red), MAP2 (green), and Hoechst (blue). Primary rat astrocyte cultures were fixed and stained for GFAP (green) and Hoechst (blue). B. CGN and cerebellar astrocyte cultures were subfractionated into mitochondrial and cytoplasmic fractions using differential centrifugation as described under “Experimental Procedures.” Protein lysates from the mitochondrial and cytosolic fractions were resolved using SDS-PAGE and immunoblotted for DIC and OGC. Immunoblotting for β-tubulin was used as a measure of fraction purity.
3.4.2 Inhibition of DIC but Not OGC Specifically Reduces the Mitochondrial GSH Pool in CGNs

We next examined the effects of butylmalonate and phenylsuccinate, DIC and OGC inhibitors, respectively, on mitochondrial GSH levels within CGNs. Dose responses were initially completed in order to establish a maximal non-cytotoxic level of each of these inhibitors (data not shown). Accordingly, CGNs were treated with 5 mM butylmalonate or 5 mM phenylsuccinate overnight. After subfractionation of CGNs into mitochondrial and cytoplasmic fractions, GSH was quantified using a DTNB colorimetric assay. CGNs showed a significant reduction of the mitochondrial GSH pool when treated with butylmalonate, but they displayed no decrement in the cytosolic pool (Fig. 3.2). Interestingly, phenylsuccinate had no discernible effect on either the mitochondrial or cytosolic pools of GSH within CGNs (Fig. 3.2). These results are consistent with the immunoblot analysis, which demonstrated that the CGNs expressed DIC but not OGC (Fig. 3.1.B). Immunoblot analysis of the subcellular fractions for cytochrome c oxidase IV and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) verified the purity of the mitochondrial and cytosolic fractions, respectively (Fig. 3.2). Furthermore, mitochondrial GSH comprised 14.3 +/- 2.7% of total cellular GSH at a concentration of 6.9 +/- 1.10 nmol/mg, consistent with previous studies (mean +/- S.E., n = 6) (Ravindranath et al., 1989; Sagara et al., 1993; Huang and Philbert, 1995; Wilkins et al., 2012).
Figure 3.2 Inhibition of DIC but not OGC specifically reduces the mitochondrial GSH pool in CGNs. CGN cultures were treated with vehicle (DMSO; Con), 5 mM butylmalonate (BM), or 5 mM phenylsuccinate (PS) overnight. Cells were then subfractionated into mitochondrial and cytoplasmic fractions using differential centrifugation. Mitochondrial and cytosolic fractions were measured for total GSH content using a DTNB assay. Data are represented as a percentage of control GSH. **, p < 0.01 compared with control; NS, not significant; n = 4 experiments with each treatment performed in duplicate. Protein lysates were resolved using SDS-PAGE and immunoblotted for cytochrome c oxidase IV (Cox-IV) and GAPDH to indicate pure fractionations. Error bars, S.E.
3.4.3 Inhibition of DIC or OGC Function Specifically Decreases the Mitochondrial GSH Pool in Cerebellar Astrocytes

In order to resolve the mitochondrial GSH transporters utilized in cerebellar astrocytes, these cultures were incubated overnight with 5 mM butylmalonate or 5 mM phenylsuccinate, after which the cerebellar astrocytes were subfractionated, and GSH was quantified as described above. Cerebellar astrocytes displayed a significant level of mitochondrial GSH depletion with phenylsuccinate but no effect of this inhibitor on the cytosolic pool of GSH (Fig. 3.3). Although the results for butylmalonate did not reach statistical significance, the trend toward mitochondrial GSH depletion with this inhibitor is in agreement with the previous immunoblot analysis showing that these astrocytes express both DIC and OGC (Fig. 3.1.B). Furthermore, mitochondrial GSH comprised 13.4 +/- 3.9% of total cellular GSH at a concentration of 2.0 +/- 0.78 nmol/mg, consistent with previous studies (mean +/- S.E., n=4) (Ravindranath et al., 1989; Sagara et al., 1993; Huang and Philbert, 1995). Cytochrome c oxidase IV blots showed consistent mitochondrial fractions. Although immunoblotting indicated that the mitochondrial phenylsuccinate sample contains GAPDH, this finding alone does not definitively demonstrate cytosolic contamination of this fraction. For instance, a previous study showed that GAPDH can localize to the mitochondria under conditions of mitochondrial permeability transition pore activation (Tarze et al., 2007). Given the significant depletion of mitochondrial GSH observed with phenylsuccinate, this mitochondrial perturbation may be occurring in astrocytes. Second, if the phenylsuccinate mitochondrial
samples did indeed contain significant cytosolic contamination, then we would expect to see a marked increase in mitochondrial GSH compared with controls because the cytosolic GSH pool is much larger than the mitochondrial GSH pool. Instead, we observe the opposite effect, where phenylsuccinate significantly reduces the mitochondrial GSH pool in cerebellar astrocytes (Fig. 3.3).
Figure 3.3 Inhibition of DIC or OGC function specifically decreases the mitochondrial GSH pool in cerebellar astrocytes

Cerebellar astrocyte cultures were treated with vehicle (DMSO; Con), 5 mM butylmalonate (BM), or 5 mM phenylsuccinate (PS) overnight. Cells were then subfractionated into mitochondrial and cytoplasmic fractions using differential centrifugation. Mitochondrial and cytosolic fractions were measured for total GSH content using a DTNB assay. Data are represented as a percentage of control GSH. **, p < 0.01 compared with control; NS, not significant; n= 3 experiments with each treatment performed in duplicate. Protein lysates were resolved using SDS-PAGE and immunoblotted for cytochrome c oxidase IV (Cox-IV) and GAPDH to indicate pure fractions. Error bars, S.E.
3.4.4 Inhibition of DIC-dependent Mitochondrial GSH Transport Renders CGNs More Susceptible to Oxidative Stress Induced by HA14-1

The unique dependence of CGNs on DIC-dependent transport provided a valuable model for examining the effects of specific inhibition of mitochondrial GSH transport on neuronal sensitivity to oxidative stress. To this end, CGNs were preincubated overnight with either 5 mM phenylsuccinate or 5 mM butylmalonate. Incubation with these doses of inhibitors are not cytotoxic on their own (Fig. 3.4.A (BM panels) and B (quantification)). Next, CGNs were treated for an additional 4 h with 12.5 μM HA14-1, alone or in combination with butylmalonate or phenylsuccinate. We have previously shown that HA14-1 causes GSH-sensitive intrinsic apoptosis and mitochondrial oxidative stress, through the inhibition of Bcl-2 in CGNs (Zimmermann et al., 2007). Therefore, we utilized this Bcl-2 homology-3 domain (BH3) mimetic, HA14-1, as a model of oxidative stress. Incubation with 12.5 μM HA14-1 alone for 4 h had no significant effect on CGN survival (Fig. 3.4.A (HA14 panels) and B (quantification)). However, inhibition of mitochondrial GSH transport through the DIC using butylmalonate rendered the CGNs significantly more sensitive to this oxidative stressor (Fig. 3.4.A (BM + HA14 panels) and B (quantification)). In contrast, preincubation with the OGC inhibitor, phenylsuccinate, did not significantly impact the susceptibility of CGNs to oxidative stress induced by HA14-1, again consistent with our data showing that CGNs do not express OGC (Fig. 3.4.B, quantification).
Figure 3.4 *Inhibition of DIC-dependent mitochondrial GSH transport renders CGNs more susceptible to oxidative stress induced by HA14-1.* A. CGN cultures were treated with vehicle (Con) or 5 mM butylmalonate (BM) overnight. The following day, cells were either left untreated (BM or Con) or treated with 12.5 μM HA14-1 (HA14 or BM_HA14) for 4 h. After treatment, cells were fixed, and β-tubulin (FITC; green) and nuclei (Hoechst; blue) were stained (top four panels). The bottom panels show decolorized nuclei from separate fields. Scale bar, 10 μm. B. Cells were treated as in A,
with the addition of a 5mM phenylsuccinate pretreatment and HA14-1 (PS+HA14) or no treatment (PS) the following day. Cells were fixed, and nuclei were stained with Hoechst. Apoptosis was quantified by determining the percentage of cells with condensed and/or fragmented nuclei. **p<0.01 versus butylmalonate alone; n = 4 experiments with each treatment performed in duplicate. Error bars, S.E. I acknowledge Danielle Kirchhof for assistance in generating the data for this figure.
3.4.5 Inhibition of DIC-dependent Mitochondrial GSH Transport Renders CGNs More Susceptible to Nitrosative Stress Induced by the Nitric Oxide Donor, SNP

We next examined the effects of selective mitochondrial GSH depletion, through the inhibition of mitochondrial GSH transport, on the vulnerability of CGNs to nitrosative stress. As described above, CGNs were preincubated overnight with either 5 mM phenylsuccinate or 5 mM butylmalonate. Incubations with these doses of inhibitors were not cytotoxic to CGNs on their own (Fig. 3.5.A, BM panel and PS panel). Next, CGNs were treated for an additional 6 h with the nitric oxide donor SNP, at a concentration (25 μM) that was also not cytotoxic on its own (Fig. 3.5.A, SNP panel). Some CGNs were also treated with SNP in combination with butylmalonate or phenylsuccinate. Consistent with the results described above for HA14-1, preincubation with butylmalonate but not phenylsuccinate rendered CGNs more susceptible to nitrosative stress-induced apoptosis (Fig. 3.5.A, compare BM + SNP and PS + SNP panels). Quantification of these data showed a statistically significant increase in CGN apoptosis induced by SNP in cells preincubated with butylmalonate versus cells incubated with butylmalonate alone (Fig. 3.5.B).
Figure 3.5 Inhibition of DIC-dependent mitochondrial GSH transport renders CGNs more susceptible to nitrosative stress induced by the nitric oxide donor, SNP. A. CGN cultures were treated with vehicle (Con), 5 mM phenylsuccinate (PS), or 5 mM butylmalonate (BM) overnight. The following day cells were either left untreated (Con, PS, or BM) or treated with 25 μM SNP (SNP, PS +SNP, or BM +SNP) for 6 h. Cells were then fixed and stained with Hoechst dye. Scale bar, 10 μm. B. cells were treated as in A, and apoptosis was quantified by determining the percentage of condensed and/or fragmented nuclei. **p<0.01 versus butylmalonate alone; n=4 experiments with each treatment performed in duplicate. Error bars, S.E. I acknowledge Danielle Kirchhof for assistance in generating the data for this figure.
3.4.6 Inhibition of DIC-dependent Mitochondrial GSH Transport Does Not Render CGNs More Susceptible to Apoptosis Induced by Deprivation of Depolarizing Potassium

Previous studies have shown that removing depolarizing potassium (5K; 5 mM potassium) induces CGN apoptosis and that this insult is insensitive to the addition of GSH-MEE(D’Mello et al., 1993; Zimmermann et al., 2005). Therefore, to determine if incubation of CGNs with the DIC inhibitor, butylmalonate, nonspecifically enhances the sensitivity of CGNs to any toxic insult, we examined the effects of 5K within this system. Incubation with 5K alone for 10 h had a significant deleterious effect on CGN survival (Fig. 3.6.A(5K panel) and B (quantification)). However, inhibition of mitochondrial GSH transport through the DIC, using butylmalonate, did not render the CGNs more sensitive to 5K (Fig. 3.6.A (BM+5K panel) and B (quantification)). Therefore, inhibition of GSH transport through the DIC does not indiscriminately render CGNs more susceptible to GSH-insensitive insults.
Figure 3.6 Inhibition of DIC-dependent mitochondrial GSH transport does not render CGNs more susceptible to apoptosis induced by deprivation of depolarizing potassium. A. CGN cultures were treated with vehicle (Con) or 5 mM butylmalonate (BM) overnight. The following day, medium was either changed to 25mM potassium minus serum (Con or BM) or changed to 5 mM potassium minus serum (5K or BM+5K). Cells were then fixed, and nuclei (Hoechst; blue) were stained. Panels show decolorized nuclei from representative fields. Scale bar, 10 μm. B. cells were treated as in A, after which cells were fixed, and nuclei were stained with Hoechst. Apoptosis was quantified by determining the percentage of cells with condensed and/or fragmented nuclei. ## p < 0.01 versus butylmalonate alone; ** p < 0.01 versus control; NS, not significant; n = 3 experiments with each treatment performed in duplicate. Error bars, S.E.
3.4.7 Effects Induced by Inhibition of DIC-dependent Mitochondrial GSH Transport

Next, we used a cell-permeable form of GSH, GSH monoethylester, to determine if the sensitization of CGNs to oxidative and nitrosative stress induced by the inhibition of DIC-dependent mitochondrial GSH transport was indeed due to the observed reduction in mitochondrial GSH (see Fig. 3.2). CGNs were preincubated with 5mM butylmalonate overnight, after which they were treated with either 12.5 μM HA14-1 (for 4 h) or 25 μM SNP (for 6 h) in the absence or presence of 2mM GSH monoethylester. Again, incubation with butylmalonate, SNP, and HA14-1 individually did not induce significant levels of apoptosis compared with control conditions (Fig. 3.7.B). However, as shown previously, preincubation with butylmalonate induced significant susceptibility to both oxidative and nitrosative stress caused by these insults (Fig. 3.7.A (BM+HA14 panels) and B (quantification)). Moreover, the cell-permeable form of GSH significantly protected CGNs from the sensitizing effects induced by inhibition of DIC-dependent mitochondrial GSH transport (Fig. 3.7.A BM + HA14 + GSHMEE panels). Quantification of these data showed statistically significant protection upon the addition of GSH monoethylester, when compared with the sensitizing conditions of pretreatment with butylmalonate and the subsequent addition of HA14-1 or SNP (Fig. 3.7.B).
Figure 3.7 A cell-permeable form of GSH protects CGNs from the sensitizing effects induced by inhibition of DIC-dependent mitochondrial GSH transport. A. CGN cultures were treated with vehicle (Con) or 5 mM butylmalonate (BM) overnight. The following day, cells were either left untreated (Con or BM) or treated with 12.5 μM HA14-1 in the presence or absence of 2 mM GSH monoethylester (GSHMEE) for 4 h, after which cells were fixed, and nuclei (Hoechst; blue) were stained. The left panels show decolorized nuclei from representative fields. The right panels show corresponding bright field images. Scale bar, 10 μm. B. Cells were treated as in A; in addition, some cells were
incubated with 25 μM SNP in the presence or absence of 2 mM GSH monoethylester. Cells were fixed, and nuclei were stained with Hoechst. Apoptosis was quantified by determining the percentage of cells with condensed and/or fragmented nuclei. **p < 0.01 versus butylmalonate alone; + p < 0.05 versus butylmalonate + SNP; ## p < 0.01 versus butylmalonate + HA14-1; n = 3 experiments with each treatment performed in duplicate. Error bars, S.E. I acknowledge Danielle Kirchhof for assistance in generating the data for this figure.
3.4.8 The Addition of Malate Fails to Protect CGNs from the Sensitizing Effects Induced by Inhibition of DIC-dependent Mitochondrial GSH Transport

Mitochondrial anion transporters work in concert with one another in order to transport critical molecules into and out of the mitochondria for energy production. Previous studies have shown that the DIC has the ability to transport malate bidirectionally in exchange for phosphate (Mizuarai et al., 2005). However, the tricarboxylate carrier transports malate into the mitochondria in exchange for citrate. Therefore, the DIC and the tricarboxylate carrier work in tandem, where DIC transports malate out of the mitochondria and the tricarboxylate carrier transports malate into the mitochondria (Kajimoto et al., 2005). These transport processes are critical for cell survival because citrate is a crucial component of fatty acid synthesis within the cytosol, whereas malate is required for the citric acid cycle in the mitochondrial matrix. Therefore, an important control experiment when inhibiting DIC-dependent transport function is to examine if malate is capable of protecting CGNs from the sensitizing effects of butylmalonate. This experiment is necessary to demonstrate that the sensitizing effects induced by inhibiting DIC-dependent transport function are not due to disruption of the malate transport capacity of the DIC. Here, we preincubated CGNs with 5mM butylmalonate in the absence or presence of 5 mM malate. Following these preincubations, CGNs were then treated for 6 h with 25 μM SNP. In the presence of malate, preincubation with butylmalonate and subsequent SNP treatment induced an even greater amount of CGN apoptosis as compared with butylmalonate in the presence of SNP alone (Fig. 3.8.A, compare BM+ SNP panel with BM +MA + SNP panel).
However, preincubation with malate alone followed by SNP treatment did not increase apoptosis beyond control levels (Fig. 3.8.A, MA +SNP panel). Quantification of these data showed a statistically significant increase in vulnerability to nitrosative stress after CGNs were preincubated with both malate and butylmalonate compared with butylmalonate preincubation alone (Fig. 3.8.B).
**Figure 3.8** The addition of malate fails to protect CGNs from the sensitizing effects induced by inhibition of DIC-dependent mitochondrial GSH transport. A. CGN cultures were treated with vehicle (Con), 5 mM butylmalonate (BM), or 5 mM malate (MA) overnight. The following day, cells were either left untreated or were treated with 25 _μM_ SNP (SNP, BM+SNP, MA+SNP, or BM+MA+SNP) for 6 h, after which cells were fixed, and nuclei (Hoechst; blue) were stained. Scale bar, 10 μm. B. cells were treated as in A. Apoptosis was quantified by determining the percentage of cells with condensed and/or fragmented nuclei. **p<0.01 versus SNP alone; ## p<0.01 versus butylmalonate+SNP; n =5 experiments with each treatment performed in duplicate. Error bars, S.E.
3.4.9 Adenoviral siRNA-mediated Knockdown of DIC Renders CGNs More Susceptible to Oxidative Stress

Finally, we utilized two distinct adenoviral constructs encoding siRNA against rat DIC and a scrambled control construct to examine their effects on neuronal vulnerability to oxidative stress. After 48 h of infection with the adenoviral constructs, CGNs were treated with 12.5 μM HA14-1 for 4 h. In addition, a parallel group of cells from each adenoviral infection was lysed and analyzed for DIC protein levels. As expected, the siRNA DIC adenoviral constructs consistently induced a significant knockdown of DIC expression compared with CGNs infected with the scrambled control (Fig. 3.9.A, siRNA1, 33.6+/−16%, n=3; siRNA2, 48+/−11%, n =9; mean +/-S.E., reduction in DIC protein level normalized to β-tubulin). Neither of the adenoviral constructs caused any significant apoptosis on their own (Fig.3.9.B, Scram, siRNA1, and siRNA2 panels). However, CGNs infected with either the DIC siRNA1 or DIC siRNA2 adenovirus and subsequently treated with HA14-1 showed a significant increase in apoptosis compared with CGNs infected with the scrambled control virus and then incubated with HA14-1 (Fig. 3.9.B, compare Scram +HA14 with siRNA1 + HA14 and siRNA2 +HA14 panels). Quantification of these data showed that knockdown of DIC with either of two distinct adenoviral siRNA constructs significantly sensitized CGNs to oxidative stress-induced apoptosis when compared with infection with the scrambled control construct (Fig. 3.9.C).
Figure 3.9 Adenoviral siRNA-mediated knockdown of DIC renders CGNs more susceptible to oxidative stress

A. CGNs were infected for 48 h with a scrambled (Scram) or two distinct DIC-specific siRNA (siRNA1 or siRNA2) adenoviruses. Cells were lysed; proteins were resolved by SDS-PAGE and immunoblotted for DIC (upper blots) and β-tubulin (lower blots).

B. CGNs were infected as described in A, after which cells were then either left untreated (Scram, siRNA1, or siRNA2) or treated with 12.5 μM HA14-1.
(Scram+HA14, siRNA1+HA14, or siRNA2 +HA14) for 4 h. Cells were fixed, and nuclei were stained with Hoechst. The panels show representative fields of decolorized nuclei. Arrowheads indicate apoptotic nuclei. Scale bar, 10 μm. C. cells wereinfected/treated as in B. and apoptosis was quantified as the percentage of cells with condensed and/or fragmented nuclei. *p<0.05 versus scrambled+HA14; ** p<0.01 versus scrambled+HA14; n=3 experiments with each treatment performed in triplicate. Error bars, S.E.
3.5 Discussion

Given that previous studies have demonstrated enhanced sensitivity of both neurons and astrocytes to specific depletion of mitochondrial GSH it is perhaps not surprising that inhibition of mitochondrial GSH transport would produce similar effects (Wüllner et al., 1999; Muyderman et al., 2007). However, it is striking that the discrete inhibition of a single inner membrane transport protein, DIC, is sufficient to significantly sensitize neurons to both oxidative and nitrosative stress. We chose to use CGNs as a model for inhibition of mitochondrial GSH transport based on their apparent dependence on DIC-mediated GSH transport. Interestingly, a mere ~32% reduction in mitochondrial GSH through specific inhibition of DIC leads to a dramatic increase in the susceptibility of CGNs to both oxidative and nitrosative stress-induced apoptosis. We used both chemical inhibition of DIC (butylmalonate) and molecular knockdown of DIC (adenoviral siRNA) in order to show that discrete inhibition of this single transporter could render CGNs more susceptible to apoptosis. As discussed previously, the mitochondrial GSH pool is an indispensable reservoir of this critical antioxidant. Several studies have shown that specific depletion of mitochondrial GSH versus depletion of the cytosolic GSH pool leads to neuronal degeneration as well as increased vulnerability of astrocytes to both oxidative and nitrosative stress (Wüllner et al., 1999; Muyderman et al., 2007). Furthermore, depletion of mitochondrial GSH may occur upstream of mitochondrial dysfunction and oxidative stress, suggesting that reductions in mitochondrial GSH may act as a trigger for these pathological mechanisms (Merad-Boudia et al., 1998). Based on our data, it becomes an interesting possibility that
dysfunction of one or more mitochondrial GSH transport proteins could lead to the enhanced susceptibility of neurons in the CNS to oxidative and nitrosative stress. This may be particularly relevant within chronic neurodegenerative diseases (e.g. PD or ALS), which have been hypothesized to occur via multiple “hits” involving gene-environment interactions as underlying pathological mechanisms (Sulzer, 2007). To date, a role for DIC or OGC dysfunction in neurodegeneration has not been examined.

In the context of neurodegenerative diseases, the devastating triad of GSH depletion, mitochondrial dysfunction, and oxidative stress has been implicated in the underlying pathology of a large number of disorders, such as Alzheimer disease, PD, and ALS (Castellani et al., 2002; Mattiazzi et al., 2002; Eckert et al., 2003; Lin and Beal, 2006; Henchcliffe and Beal, 2008; Murata et al., 2008). Specifically, GSH has been shown to be depleted within the substantia nigra of PD patients (Perry et al., 1986). Moreover, depletion of nigral GSH was detected in a mouse model of PD before the onset of complex I deficiency and neurodegeneration (Chinta et al., 2007). Similarly, in a familial mouse model of ALS, the G93A mutant hSOD1 mouse, GSH depletion in the spinal cord was positively correlated with disease onset and progression (Chi et al., 2007). Forced reduction of GSH levels in the G93A mutant hSOD1 mouse model, through the knockdown of γ-glutamyl cysteine ligase modifier subunit (the rate-limiting enzyme in GSH synthesis) led to a decrease in life span, increased oxidative stress, and accelerated motor dysfunction (Vargas et al., 2011). Furthermore, in sporadic ALS patients, erythrocyte GSH levels were found to be diminished, whereas lipid peroxidation levels were significantly increased compared with healthy age-matched controls (Babu et
al., 2008). Despite this abundant and compelling evidence for a key role of GSH depletion within neurodegenerative diseases, neither mitochondrial GSH nor mitochondrial GSH transport mechanisms explicitly have been studied within models of these diseases.

To further support the role of DIC-dependent mitochondrial GSH transport as a protective mechanism in neurons, while excluding the contribution of interference with the malate transport function of DIC, we examined whether malate would protect CGNs from the sensitizing effects of DIC inhibition to nitrosative stress. Our results show that the addition of malate in the presence of the DIC inhibitor butylmalonate caused CGNs to become more susceptible to nitrosative stress than in the presence of butylmalonate alone. This result can be attributed to the competitive inhibition produced by butylmalonate and malate together on DIC-dependent mitochondrial GSH transport. Butylmalonate inhibits DIC through competitive inhibition by binding to the transport site, and because it is an analog, it does not become transported or metabolized (Lash, 2006; Palmieri et al., 1971). Therefore, the addition of malate with butylmalonate at the same concentration would allow malate to outcompete butylmalonate for transport. However, by adding both of these chemicals together, GSH would have to compete with both malate and butylmalonate, therefore causing an even more significant reduction in mitochondrial GSH transport and a greater vulnerability to nitrosative stress. In contrast to malate addition, the sensitizing effects induced by DIC inhibition with butylmalonate could be rescued by adding back GSH as a cellpermeable esterified form. Therefore, because malate was unable to protect CGNs from the enhanced susceptibility induced by
DIC inhibition, whereas adding back GSH did prevent these effects, we are able to rule out deficits in fatty acid synthesis and the citric acid cycle as the mechanism by which CGNs become vulnerable to apoptosis, leaving mitochondrial GSH transport as the most probable mechanism. Depletion of mitochondrial GSH and increased susceptibility to oxidative and nitrosative stress did not occur in CGNs exposed to phenylsuccinate, ruling out the possibility that chemical analogs of commonly transported anions are capable of non-specifically inducing neuronal susceptibility to these insults. Furthermore, butylmalonate did not render the CGNs more vulnerable to a GSH-insensitive insult, 5K-induced apoptosis. The specificity of the sensitizing effects of butylmalonate is also supported through the use of two distinct adenoviral siRNAs to knock down DIC, which similarly induced increased susceptibility to oxidative stress compared with a scrambled control virus.

Previous studies have shown that whole brain mitochondria utilize the tricarboxylate carrier (Slc25a1) as a mitochondrial GSH transport protein, whereas mitochondria isolated specifically from cerebral cortex, which express both DIC and OGC, use primarily DIC as a mitochondrial GSH transport protein (Wadey et al., 2009; Kamga et al., 2010). Our study shows that CGNs are dependent on DIC, whereas cerebellar astrocytes use both DIC and OGC as mitochondrial GSH transport proteins. CGNs appear to be unique because they are insensitive to phenylsuccinate; however, based on the relatively small decrease in mitochondrial GSH (~32%) observed with butylmalonate, it cannot be ruled out that a second inner membrane transporter, such as the tricarboxylate carrier, is also being used to transport GSH into CGN mitochondria.
Overall, more studies are necessary to determine the identity of a second transport protein that contributes significantly to mitochondrial GSH transport within CGNs.

Several studies have shown that mitochondrial oxidative stress plays a key role within neurodegenerative diseases, such as PD and ALS. In one such study, a partial knockdown of the mitochondrial specific manganese-superoxide dismutase (SOD2) led to a significant decrease in survival and a greater decline in motor function within the G93A mutant hSOD1 mouse model (Andreassen et al., 2000). In addition, overexpression of SOD2 in a differentiated human neuroblastoma cell line protected from cell death induced by the overexpression of several different mutant SOD1 proteins (Flanagan et al., 2002). These studies indicate the importance of maintaining the mitochondrial redox status for neuronal viability. Mitochondrial SOD2 and mitochondrial GSH (via the activity of GSH peroxidase) work in concert to abolish the free radicals generated by leakage from the electron transport chain, and these antioxidants are equally important in maintaining the redox balance within mitochondria. Therefore, the prior mentioned studies focused on SOD2, in combination with our data, indicate that the antioxidant repertoire of mitochondria is critical in protecting neurons from pathological mechanisms common to many neurodegenerative diseases.

In addition to defining the specific transport proteins involved in mitochondrial GSH transport in the CNS, the regulation of mitochondrial GSH transport is also an area that is not well understood. We have previously shown that Bcl-2 plays a central role in maintaining the mitochondrial GSH pool in CGNs and is also capable of directly binding GSH(Zimmermann et al., 2007). Furthermore, Bcl-2 is an interacting partner of OGC,
and co-overexpression of these proteins leads to a significant increase in mitochondrial GSH (Wilkins et al., 2012). Finally, BH3-only proteins (e.g. Bim) and BH3 mimetics antagonize the Bcl-2/GSH interaction and inhibit GSH transport into isolated rat brain mitochondria (Zimmermann et al., 2007; Wilkins et al., 2012). Therefore, Bcl-2 appears to play a crucial role in regulating mitochondrial GSH transport. This point becomes particularly relevant when considering that Bcl-2 function is often compromised within neurodegenerative diseases. For instance, Bcl-2 expression has been shown to be significantly decreased within the spinal cord of ALS patients (Mu et al., 1996). In the G93A mutant hSOD1 mouse model of familial ALS, the mutant SOD1 protein was found to interact with Bcl-2 and form dysfunctional aggregates with Bcl-2 at mitochondria (Pasinelli et al., 2004). This interaction between G93A mutant SOD1 and Bcl-2 was also shown to induce a toxic conformational change in Bcl-2, which prevents it from performing its anti-apoptotic function (Pedrini et al., 2010). Therefore, it would be of interest to determine if these deficits in Bcl-2 protein expression or its interaction with G93A mutant SOD1 lead to consequential decreases in mitochondrial GSH transport in the context of specific neurodegenerative diseases like ALS.

In summary, we have shown that by modulating mitochondrial GSH transport through the specific inhibition of a discrete inner membrane transporter, DIC, neurons become more vulnerable to apoptosis induced by oxidative or nitrosative stress. This study provides novel insights into the importance of mitochondrial GSH transport in maintaining neuronal survival and suggests that understanding the mechanisms involved
in the regulation of mitochondrial GSH transport in neurons may reveal novel therapeutic targets for neurodegenerative disorders.
CHAPTER FOUR: STABLE OVEREXPRESSION OF THE 2-OXOGLUTARATE CARRIER ENHANCES NEURONAL CELL RESISTANCE TO OXIDATIVE STRESS VIA BCL-2-DEPENDENT MITOCHONDRIAL GSH TRANSPORT

4.1 Abstract

4.1.1 Aims

Mitochondrial glutathione (GSH) is a key endogenous antioxidant pool and its maintenance is critical for cell survival. Here, we generated stable NSC34 motor neuron-like cell lines overexpressing the mitochondrial GSH transporter, the 2-oxoglutarate carrier (OGC), to further elucidate the importance of mitochondrial GSH transport in determining neuronal resistance to oxidative stress.

4.1.2 Results

Two stable OGC cell lines displayed specific increases in mitochondrial GSH content and resistance to oxidative and nitrosative stressors, but not staurosporine. Inhibition of transport through OGC and reduced levels of mitochondrial GSH re-sensitized the stable cell lines to oxidative stress. The stable OGC cell lines displayed significant upregulation of the anti-apoptotic protein, Bcl-2. This result was reproduced in parental NSC34 cells by chronic treatment with GSH monoethylester, which specifically increased mitochondrial GSH levels. Knockdown of Bcl-2 expression with siRNA decreased mitochondrial GSH and re-sensitized the stable OGC cells to oxidative stress.
Finally, endogenous OGC was co-immunoprecipitated with Bcl-2 from rat brain lysates in a GSH dependent manner.

4.1.3 Innovation

These data are the first to show that increased mitochondrial GSH transport is sufficient to enhance neuronal resistance to oxidative stress. Moreover, sustained and specific enhancement of mitochondrial GSH leads to increased Bcl-2 expression, a required mechanism for the maintenance of increased mitochondrial GSH levels.

4.1.4 Conclusion

Modulation of mitochondrial GSH transport renders neuronal cells resistant to oxidative stress, in a manner dependent on Bcl-2. These findings suggest that mitochondrial GSH transport may be deficient in neurodegenerative diseases where Bcl-2 expression and function have been shown to be compromised.

4.2 Introduction

Mitochondrial dysfunction and oxidative stress, particularly at the level of the mitochondria, play key roles in the pathogenesis underlying several neurodegenerative diseases (Lin and Beal, 2006). While the central nervous system (CNS) accounts for a mere two percent of total body weight, it generates approximately twenty percent of the resting metabolic rate (Silver and Erecinska, 1998). Furthermore, neurons require high amounts of ATP production, most of which is generated from oxidative phosphorylation at the level of the mitochondrial electron transport chain (ETC), and are thus particularly vulnerable to reactive oxygen species (ROS) generated by electron leakage from the ETC.
(Kann and Kovács, 2007). If ROS production is not balanced by free radical scavenging systems, such as GSH or thioredoxin, then mitochondrial oxidative stress (MOS) occurs triggering damage to DNA, lipids and proteins, ultimately leading to apoptotic or necrotic degeneration of neurons (Lin and Beal, 2006).

Glutathione (GSH), an endogenous tripeptide antioxidant, is the most prominent cellular thiol present at concentrations which are 500-1000 times greater than other free radical scavenging systems (Schafer and Buettner, 2001; Filomeni et al., 2002). There are several reservoirs of GSH throughout the cell, but in particular the mitochondrial GSH pool has been shown to be critical for cell survival in several different systems (Meredith and Reed 1982; Meredith and Reed 1983; Muyderman et al., 2007; Wilkins et al., 2013). GSH is compartmentalized into mitochondria via a facilitated transport process involving inner mitochondrial anion transporters such as the dicarboxylate (Slc25a10; DIC), 2-oxoglutarate (Slc25a11; OGC), and tricarboxylate (Slc25a1; TCC) carriers (Lash, 2006; Wadey et al., 2009; Kamga et al., 2010). We have previously shown that mitochondrial GSH transport is critical for cell survival in primary cerebellar granule neurons (CGNs); discrete inhibition or molecular knockdown of a single mitochondrial GSH transporter (DIC) significantly sensitized these neurons to both oxidative and nitrosative stress (Wilkins et al., 2013). These previous findings indicate that not only is the mitochondrial GSH pool critical, but that mitochondrial GSH transport is a major determinant of neuronal susceptibility to oxidative stress.

To further elucidate the importance of mitochondrial GSH transport on neuronal viability, we derived NSC34 motor neuron-like cell lines that stably overexpress V5-
tagged OGC. Here, we show that the stable OGC cell lines display a specific increase in mitochondrial GSH levels. In addition, these OGC stable cell lines are significantly resistant to oxidative and nitrosative stress, as well as to a GSH depleting agent, ethacrynic acid, but not to a classically Bax-dependent apoptotic inducer, staurosporine. Furthermore, the stable OGC cell lines showed a significant upregulation of Bcl-2 protein expression, an effect that is dependent on enhanced mitochondrial GSH levels. Finally, either chemical inhibition of OGC transport function or knockdown of Bcl-2 using siRNA, led to a decrease in mitochondrial GSH levels and re-sensitization of the stable OGC cell lines to oxidative stress. It has been previously shown in several systems using either recombinant proteins or transient transfection that Bcl-2 and OGC can interact in a GSH-dependent manner (Gallo et al., 2011; Wilkins et al., 2012). However, here we provide the first evidence that endogenous Bcl-2 and OGC interact in a GSH-dependent manner in lysates derived from rat brain. Overall, these findings suggest a synergistic mechanism between Bcl-2 and OGC in facilitating mitochondrial GSH transport and further establish the importance of mitochondrial GSH transport in sustaining neuronal viability under conditions of increased oxidative and nitrosative stress.

4.3 Materials and Methods

4.3.1 Reagents

Phenylsuccinic acid, L-reduced glutathione, ethacrynic acid, staurosporine, primary antibody against α-tubulin, and GSH monoethylester were purchased from Sigma-Aldrich (St.Louis, MO). The MTT cell viability assay, Geneticin (G418) and
Lipofectamine were obtained from Invitrogen (Carlsbad, CA). The GSH assay was purchased from Oxford Biomedical (Rochester Hills, MI). The mitochondrial/cytosolic fractionation kit was from Biovision (Mountain View, CA). Antibody against cytochrome c oxidase IV (Cox-IV) was purchased from Cell Signaling (Beverly, MA). Anti-glyceraldehyde 3-phosphate dehydrogenase (Anti-GAPDH), anti-Bcl-2, anti-OGC, non-immune IgG, anti- neuron specific β3 tubulin and anti-V5 antibodies were purchased from Abcam (Cambridge, MA). Sodium nitroprusside was purchased from Calbiochem (Billerica, MA). Secondary antibodies for immunoblotting and reagents for enhanced chemiluminescence were obtained from GE Life Sciences (Piscataway, NJ).

DharmaFECT Transfection Kit, Smart pool siRNA against mouse Bcl-2, and Non-target siRNA were purchased from Thermoscientific Pierce Biotechnology (Rockford, IL). Optimem and Dulbecco’s Modified Eagle’s Medium (DMEM) were obtained from Gibco (Carlsbad, CA). V5-OGC plasmid was a generous gift from Dr. Larry Lash (Wayne State University). NSC34 cells were kindly provided by Dr. Neil Cashman (University of British Columbia).

4.3.2 Establishment of stable OGC NSC34 cell lines

NSC34 cells were plated at 80% confluence in 10 cm cell culture dishes, and transfected with 40 μg of V5-OGC plasmid DNA using a standard Lipofectamine 2000 protocol. At 24 h post transfection cells were split into two cell culture dishes and the following day 500 μg/mL of G418 was added to the media to allow for the selection of transfected cells. Media was changed every three days with the addition of 500 μg/mL of G418. Clones were then selected and grown in a 24-well plate in duplicate, grown to
confluence and immunoblotted for V5 expression. High expressing clones were propagated for further experiments.

4.3.3 Cell culture of parental (wild-type, WT) and stable OGC NSC34 cell lines

NSC34 WT cultures were grown in DMEM high glucose medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and 100 units/ml/100 μg/ml penicillin/streptomycin. NSC34 stable OGC cell lines were grown in identical media containing 500 μg/mL of G418. Cells were plated on 6-well plates and grown to ~80% confluence at 37 °C in 10% CO₂, at which time cells were utilized for the described experiments.

4.3.4 Mitochondrial/ Cytosolic Fractionation

Cells were treated as indicated in the 4.4 Results section. Media was aspirated and cells were washed once with 1X ice-cold phosphate buffered saline (PBS), pH 7.4. One ml of cytosolic buffer, provided in the kit was diluted 1:5 in ddH₂O, with added protease inhibitor cocktail and 1 mM DTT as per the manufacturer’s recommendations. A 100 μL aliquot of the cytosolic buffer was added per well and incubated on ice for 20 min, at which point cells were harvested and homogenized with 40 passes of a Dounce homogenizer. Samples were then spun down at 720 rcf for 10 min at 4º C. The supernatant was transferred to a new tube which was labeled “mitochondrial fraction” and the supernatant was spun down at 10,000 rcf for 30 min at 4ºC. The supernatant was transferred to a new tube which was labeled the “cytosolic fraction” and the pellet in the mitochondrial fraction tube was re-suspended in 100 μL of mitochondrial buffer (provided in the kit) with protease inhibitor cocktail and 1 mM DTT as per the
manufacturer’s recommendations. Samples were immediately used for further experiments.

4.3.5 Glutathione Assay

Total glutathione (reduced GSH+oxidized glutathione (GSSG)) was measured using an assay kit (DTNB) from Oxford Biomedical, following the manufacturer’s protocol. All glutathione measurements were normalized to protein concentration.

4.3.6 Immunoblot Analysis

Protein concentrations of samples were determined by a BCA protein assay. Equal protein concentrations were subjected to SDS-PAGE, transferred and immunoblotted as previously described (Linseman et al., 2001). Briefly, PVDF membranes were incubated in blocking solution (10% BSA and 0.01% sodium azide diluted in PBS with 0.1% Tween 20; PBS-T) for 1 h at room temperature (RT). The primary antibodies were diluted in blocking solution at the manufacturer’s recommended dilution and membranes were either incubated with primary antibody for 1 h at RT or overnight at 4°C, as per manufacturer’s recommendations. Membranes were then washed 5x over 30 min with PBS-T. Next, membranes were incubated for 1 h at RT with horseradish peroxidase-conjugated secondary antibodies that were diluted to appropriate concentrations with PBS-T. Membranes were then washed 5x over 30 min with PBS-T. Proteins were detected by enhanced chemiluminescence.

4.3.7 MTT Viability Assay

After treatment as indicated in the Figure Legends or Results, cell were incubated with 100 μL of 12 mM MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
bromide) reagent for 4 h at 37°C, 10% CO₂. Next, 2 ml of dimethyl sulfoxide was added to solubilize the formazan and the plate was read at 570 nm in a spectrophotometer.

4.3.8 SMARTPOOL® siRNA mediated knockdown of Bcl-2

Stable NSC34 OGC cells were transfected with either the SMARTPOOL® siRNA against mouse Bcl-2 or non-target siRNA pool using the DharamaFECT transfection protocol. Briefly, 25 nM of appropriate siRNA per well was incubated with 5 µL of DharmaFECT transfection reagent separately with serum free medium for 5 min at RT. siRNA and DharmaFECT in serum free medium were mixed and incubated at RT for 20 min. The mixture of siRNA/DharamaFECT was then added to the cells and incubated for 48 h at 37°C and at 10% CO₂ before subsequent experiments were completed.

4.3.9 Immunoprecipitation

Whole rat brain was obtained from two P7 rats and homogenized with 8 passes of the loose pestle and 4 passes of the tight pestle from a dounce homogenizer in 0.1% Triton X-100 in lysis buffer containing protease inhibitors and 10 mM reduced GSH. The immunoprecipitation reaction consisted of 200 µg of protein lysate with 4 µg antibody to Bcl-2, OGC, or non-immune IgG; reactions were incubated overnight at 4°C with mixing by inversion. Next, 100 µL of protein A/G agarose beads were incubated with the immunoprecipitation reactions for 4 h, 4°C, mixing by inversion. Samples were spun down and washed 3x with 0.1% Triton X-100 in lysis buffer, before being resolved by SDS-PAGE and immunoblotted. Total lysate was loaded at 100 µg of protein. All animal procedures were performed according to a protocol approved by the University of Denver Institutional Animal Care and Use Committee.
4.3.10 Statistical Analysis

One-way analysis of variance (ANOVA) followed by a post hoc Tukey’s test or an unpaired Student’s T test was performed to determine statistical significance between data sets. A $p$ value of $< 0.05$ was considered to be statistically significant.

4.4 Results

4.4.1 Stable overexpression of OGC specifically increases the mitochondrial GSH pool

To establish the successful overexpression of V5-tagged OGC we used a parental (un-transfected) NSC34 cell line to compare with two separate NSC34 cell lines which showed resistance to geneticin. We immunoblotted all cell lines for both the V5 epitope tag and OGC protein levels. Both stable OGC cell lines displayed abundant expression of V5 and OGC compared to parental NSC34 cells (indicated as wild-type (WT) in Figure 4.1.A). Lysates were also blotted against β3 tubulin, a neuron specific loading control, thus indicating that the overexpression of OGC did not appear to change the neuronal phenotype of the NSC34 cells (Figure 4.1.A). Stable OGC cell lines were continually monitored for maintenance of high V5-OGC expression throughout the course of the experiments.

In order to examine the effects of stable OGC expression on mitochondrial GSH levels, stable cell lines and WT NSC34 cells were fractionated into mitochondrial and cytosolic fractions and GSH levels were measured. Both stable OGC cell lines displayed a significant and specific approximate two-fold increase in mitochondrial GSH levels compared to WT NSC34 cells (Figure 4.1.B). Mitochondrial GSH concentrations
calculated as nmoles/mg of protein were as follows, for WT, 24.4 +/- 3.4; OGC 3, 50.3 +/- 8.1; OGC 6, 57.6 +/- 14.7 (mean +/- SEM, n=6). The percentage of mitochondrial GSH compared to cytosolic GSH was in a range consistent with previous studies, where the mitochondrial GSH pool is between 5% and 30% of total cellular GSH (Lash, 2006). For WT NSC34 cells, mitochondrial GSH comprised 4.6% +/- 1.1%, while for OGC 3 mitochondrial GSH was 7.2% +/- 3.1%, and for OGC 6 mitochondrial GSH was 12.2% +/- 5.6%, of total cellular GSH (mean +/- SEM, n=7). These data further support the purity of the cellular fractionations, along with the immunoblots against cytochrome c oxidase IV (Cox-IV), a mitochondrial specific protein, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a cytosolic marker (Figure 4.1.B).
Figure 4.1 Stable overexpression of OGC specifically increases the mitochondrial GSH pool 

A. Whole cell lysates were resolved by SDS-PAGE and immunoblotted against V5, OGC and neuron specific β3 tubulin. OGC stable cell lines are indicated as OGC 3 and OGC 6; parental NSC34 cells are indicated as wild-type (WT). B. Cells were fractionated by differential centrifugation and total GSH content was then measured using a DTNB colorimetric assay. Data are represented as a percentage of WT GSH measurements. * p<0.05 versus WT mitochondrial GSH; **p<0.01 versus WT mitochondrial GSH in an unpaired student’s T-test, n=7, error bars indicate standard error. Cellular fractions were resolved using SDS-PAGE and immunoblotted against Cox-IV and GAPDH.
4.4.2 The stable OGC NSC34 cell lines are resistant to oxidative stress induced by hydrogen peroxide

To determine if the stable OGC cell lines were more resistant than WT NSC34 cells to oxidative stress, the reactive intermediate hydrogen peroxide (H$_2$O$_2$) was used. Here, we employed two doses of H$_2$O$_2$, 500 µM or 1 mM, for a period of 24 h. In Figure 4.2.A, brightfield images indicate the distinct cell death induced at both doses of H$_2$O$_2$ in the WT NSC34 cell line, and the striking resistance of both OGC stable cell lines to this oxidative stressor. In order to quantify the differences in cell viability between the WT and stable OGC cell lines, an MTT assay was employed. The WT cells displayed a significant loss of cell viability compared to the untreated controls at both 500 µM and 1 mM H$_2$O$_2$ (Figure 4.2.B). However, neither stable OGC cell line showed any significant decrease in cell viability at either dose of H$_2$O$_2$ (Figure 4.2.B). Upon completion of these experiments, it became clear that both OGC stable cell lines proliferated at a much higher frequency compared to the WT NSC34 cells. While all cell lines were plated at one million cells per well, increased proliferation of the stable OGC cell lines induced a greater cell density compared to WT NSC34 cells. This is important, because we have previously shown that increased cell density can lead to increased expression of pro-survival proteins, such as Bcl-2 and Bcl-X$_L$ (Zimmermann et al., 2005). This led us to question the effect of the high proliferation frequency of the stable OGC NSC34 cell lines on their resistance to oxidative stress.
Figure 4.2 The stable OGC NSC34 cell lines are resistant to oxidative stress induced by hydrogen peroxide. A. After plating cells at one million cells per well, cells were treated the following day with either vehicle control (Con); 500 μM H₂O₂ or 1 mM H₂O₂ overnight. Brightfield images were captured following treatment. B. Cells were plated and treated as in A, after which an MTT cell viability assay was completed. Data are represented as percent of Con for each cell line. * p<0.05 versus Con; ** p<0.01, compared to Con WT; NS, not significant via a one-way ANOVA with posthoc Tukey’s test, n=6, error bars indicate standard error. I acknowledge Samantha Brock for assistance in generating the data for this figure.
4.4.3 Resistance of OGC overexpressing cell lines to oxidative stress is not dependent on cell density

To address the above possibility, we employed three different cell plating densities to discern the dependence on cell density for resistance to oxidative stress. Brightfield images are shown to indicate the relative difference in each of the cell densities used (Figure 4.3.A). The WT NSC34 cell line showed significant loss of cell viability with treatment of H$_2$O$_2$ at each cell density tested (Figure 4.3.B). In contrast, neither stable OGC cell line showed any statistically significant decrement in cell viability in response to H$_2$O$_2$ treatment at any of the aforementioned densities (Figure 4.3.C and 4.3.D). However, there was a trend towards loss of cell viability with H$_2$O$_2$ in cells plated at 100,000 cells per well, although it did not reach statistical significance (Figure 4.3.C and 4.3.D). As indicated by the brightfield images in Figure 4.3.A, 100,000 cells per well is a very sparse density, thus some sensitivity to H$_2$O$_2$ of all three cell lines at this cell plating density should perhaps be anticipated. Furthermore, at the 250,000 and 500,000 cells per well densities this decline was not observed in either of the stable OGC cell lines (Figure 4.3.C and 4.3.D). These results indicate that the observed resistance of the OGC cell lines to H$_2$O$_2$ is not due to enhanced proliferation or higher cell density.
Figure 4.3 Resistance of OGC overexpressing cell lines to oxidative stress is not dependent on cell density

A. Cells were plated at the indicated densities and brightfield images were captured the following day. B. After plating of WT NSC34 cells at the indicated densities, cells were treated with vehicle control (Con); or the indicated concentrations of H$_2$O$_2$ for 24 h. An MTT cell viability assay was completed and data are represented as a percent of Con for each cell line. * p<0.05 versus WT Con at 100,000 cells/well; + p<0.05 versus WT Con at 250,000 cells/well ; ## p<0.01 versus WT Con at 500,000 cells/well. C. OGC 3 cells were plated and treated as in B with an MTT cell viability assay being completed. NS, not significant. D. OGC 6 cells were plated and treated as in B with an MTT cell viability assay being completed. NS, not significant. For B-D all statistical comparisons were analyzed by one-way ANOVA with posthoc tukey test, n=3, error bars indicate standard error. I acknowledge Samantha Brock for assistance in generating the data for this figure.
4.4.4 Stable overexpression of OGC prevents loss of cell viability in the presence of ethacrynic acid

Ethacrynic acid has previously been shown to non-preferentially deplete both mitochondrial and cytosolic pools of GSH, and thus induce cell death. Therefore, to determine if enhanced mitochondrial GSH transport affords protection from this GSH depleting agent, we examined the toxic effects of ethacrynic acid. Brightfield images are shown of representative fields for each cell line at both doses of ethacrynic acid used (Figure 4.4.A). The WT NSC34 cell line displayed significantly reduced cell viability following exposure to two doses of ethacrynic acid (Figure 4.4.B). In contrast, stable OGC overexpression prevented this loss of cell viability induced by ethacrynic acid (Figure 4.4.B). Therefore, enhanced mitochondrial GSH transport prevents cell death induced by the depletion of total cellular GSH levels, illustrating the key role of the mitochondrial GSH pool in maintaining neuronal survival.
Figure 4.4 Stable overexpression of OGC prevents loss of cell viability in the presence of ethacrynic acid. A. Cells were plated at one million cells per well, following which cells were treated with vehicle control (Con); 50 μM or 70 μM ethacrynic acid (EA) for 24 h. Brightfield images were captured following treatment. B. Cells were treated as in A, following which an MTT cell viability assay was completed. Data are represented as a percent of Con for each cell line. ** p<0.01 compared to WT Con; NS, not significant via a one-way ANOVA with posthoc Tukey’s test, n=4, error bars indicate standard error. I acknowledge Josie Gray for assistance in generating the data for this figure.
4.4.5 Overexpression of OGC renders NSC34 cells resistant to nitrosative stress

The nitric oxide donor, sodium nitroprusside (SNP), was used to induce nitrosative stress and determine if enhanced mitochondrial GSH transport would protect from this insult. Brightfield images taken after SNP treatment indicated that the WT NSC34 cells were sensitive to both doses of SNP used, but no toxic effect was observed in either of the OGC stable cell lines (Figure 4.5.A). The WT cells displayed a significant loss of cell viability as measured by an MTT assay at both SNP doses (Figure 4.5.B). However, neither OGC cell line showed any significant decrement in cell viability in response to SNP (Figure 4.5.B). Therefore, overexpression of the mitochondrial GSH transporter, OGC, and thus enhancement of mitochondrial GSH transport, reduces neuronal sensitivity to nitrosative stress conditions.
Figure 4.5 Overexpression of OGC renders NSC34 cells resistant to nitrosative stress. A. Cells were plated at one million cells per well, following which cells were treated with vehicle control (Con), 200 μM or 250 μM sodium nitroprusside (SNP) for 24 h. Brightfield images were captured following treatment. B. Cells were treated as in A, following which an MTT cell viability assay was completed. Data are represented as a percent of Con for each cell line. ** p<0.01 compared to WT Con; NS, not significant via a one-way ANOVA with posthoc Tukey’s test, n=4, error bars indicate standard error. I acknowledge Samantha Brock for assistance in generating the data for this figure.
4.4.6 Stable overexpression of OGC does not protect NSC34 motor neuron-like cells from staurosporine-induced apoptosis

We next examined the ability of enhanced mitochondrial GSH transport, and thus increased mitochondrial GSH content, to protect from a non-oxidative cell death mechanism. Stable OGC cell lines and WT NSC34 cells were treated with two doses of staurosporine (STS), a classically Bax-dependent method of inducing intrinsic apoptosis. WT and OGC 3 NSC34 cells both displayed the caspase-generated cleavage product of PARP in response to STS treatment, indicating an apoptotic mode of cell death (Figure 4.6.A). Furthermore, WT NSC34 cells and both stable OGC cell lines displayed similar significant decreases in cell viability at both doses of STS (Figure 4.6.B). Therefore, stable overexpression of the mitochondrial GSH transporter, OGC, did not afford protection from this classical (non-oxidative) apoptotic insult. These data demonstrate the specificity of increased mitochondrial GSH transport for enhancing the resistance of neuronal cells to oxidative damage.
Figure 4.6 Stable overexpression of OGC does not protect NSC34 motor neuron-like cells from staurosporine-induced apoptosis A. Cells were plated at one million cells per well, following which cells were treated with vehicle control (Con); 100 nM or 500 nM staurosporine (STS) for 24 h. Cells were lysed and proteins were resolved by SDS-PAGE, immunoblot against PARP was completed. FlPARP-full length PARP, Clvg-PARP-cleavage of PARP. B. Cells were treated as in A, following which an MTT cell viability assay was completed, Data are represented as a percent of Con for each cell line. ** p<0.01 compared to WT Con; *p<0.05 compared to OGC 3 or OGC 6 Con via a one-way ANOVA with posthoc Tukey’s test, n=4, error bars indicate standard error.
4.4.7 Chemical inhibition of OGC transport function specifically reduces mitochondrial GSH and re-sensitizes OGC stable cell lines to oxidative stress induced by hydrogen peroxide.

Phenylsuccinate is a chemical analog of 2-oxoglutarate that specifically blocks transport through the OGC. In order to show that enhanced mitochondrial GSH transport was responsible for conferring resistance of both stable OGC cell lines to oxidative stress, phenylsuccinate was used to inhibit OGC-dependent transport. First, we examined the effects of phenylsuccinate on mitochondrial GSH levels. Both OGC stable cell lines showed significant and specific decreases in mitochondrial GSH suggesting that phenylsuccinate successfully reduced the transport of GSH into the mitochondria (Figure 4.7.A). Following phenylsuccinate treatment, the mitochondrial GSH concentrations calculated as nmoles/mg were 29.8 +/- 5.3 for OGC 3 and 20.9 +/- 5.5 for OGC 6 (mean +/- SEM, n=3). Therefore, phenylsuccinate lowered the mitochondrial GSH concentrations in both OGC 3 and OGC 6 to approximately WT levels (24.4 +/- 3.4 as discussed in Figure 4.1). Cox-IV and GAPDH immunoblots indicate the purity of the mitochondrial fractions (Figure 4.7.A). Next, OGC stable cell lines were co-incubated with phenylsuccinate and H₂O₂, phenylsuccinate alone, or H₂O₂ alone and cell viability was measured. Following incubation with phenylsuccinate or H₂O₂ alone, neither OGC cell line showed any significant decrease in cell viability (Figure 4.7.B and 4.7.C). However, both cell lines showed significant reductions in cell viability at both doses of
H$_2$O$_2$ when OGC was simultaneously inhibited with phenylsuccinate, as compared to cells that were treated with H$_2$O$_2$ alone (Figure 4.7.B and 4.7.C).
Figure 4.7 Chemical inhibition of OGC transport function specifically reduces mitochondrial GSH and re-sensitizes OGC stable cell lines to oxidative stress induced by hydrogen peroxide. A. Stable OGC NSC34 cells were treated with 10 mM phenylsuccinate (PS) overnight. Cells were then fractionated using differential centrifugation. Cell fractions were measured for total GSH using a DTNB colorimetric assay. Data are represented as a percent of untreated control (Con) GSH. *p<0.05 compared to OGC 3 Con; **p<0.01 versus OGC 6 Con; NS, not significant via a Student’s T-test. n=4, error bars indicated standard error. Cell fractions were resolved by SDS-PAGE and immunoblotted against Cox-IV and GAPDH. B. Stable OGC 3 NSC34 cells were treated with 10 mM PS alone or in combination with 500 µM or 1 mM H₂O₂. At 24 h after treatment an MTT assay was completed and data are represented as percent of the representative Con. * p<0.05, **p<0.01 versus Con, NS, not significant; via a
one-way ANOVA with posthoc Tukey’s test n=3, error bars indicate standard error. C. Stable OGC 6 NSC34 cells were treated with 10 mM PS alone or in combination with 500 μM or 1 mM H₂O₂. At 24 h after treatment an MTT assay was completed and data are represented as percent of the representative control (Con). * p<0.05, ** p<0.01 versus Con, NS, not significant; via a one-way ANOVA with posthoc Tukey’s test n=5, error bars indicate standard error.
4.4.8 Specific increases in mitochondrial GSH induce an increase of the anti-apoptotic protein Bcl-2

Previous studies using recombinant proteins or transient overexpression constructs have shown that Bcl-2 and OGC are interacting partners (Gallo et al., 2011; Wilkins et al., 2012). Thus, Bcl-2 protein levels were examined in the OGC stable cell lines and interestingly, both lines displayed increased Bcl-2 expression, compared to WT NSC34 cells (Figure 4.8.A) (approximate 5 fold increase). In order to determine if the increased Bcl-2 expression was generally due to stable OGC overexpression or more specifically, to increased mitochondrial GSH, WT NSC34 cells were chronically treated (for 48 h) with the cell-permeable GSH monoethylester (GSH-MEE). In Figure 4.8.B, we show that chronic exposure to GSH-MEE specifically increased mitochondrial GSH levels, with no significant effect on the cytosolic pool. Cox-IV and GAPDH immunoblots indicate the purity of the mitochondrial and cytosolic fractions (Figure 4.8.B). Next, WT NSC34 cells chronically pre-treated with GSH-MEE were immunoblotted for Bcl-2 protein levels, which were significantly increased compared to untreated WT NSC34 cells (Figure 4.8.C) (approximate 2 fold increase). Overall, these data show that an upregulation of Bcl-2 protein expression occurs as a result of specifically increased mitochondrial GSH levels.
**Figure 4.8** Specific increases in mitochondrial GSH induces an increase of the anti-apoptotic protein Bcl-2. A. Lysates were resolved by SDS-PAGE and immunoblotted against Bcl-2 and α-tubulin. Densitometry was completed for Bcl-2 and was normalized to α-tubulin. Data are represented as percent of WT Bcl-2 levels. *p<0.05 versus WT via a one way ANOVA with post-hoc Tukey’s test, n=3, error bars indicate standard error. B. WT NSC34 cells were treated for 48 h with 2 mM GSH-MEE, fractionated by differential centrifugation and total GSH was measured using a DTNB colorimetric assay. Data are represented as a percent of Con GSH. **p<0.01 versus Con via an unpaired student’s T-test n=7, error bar indicate standard error. Samples were resolved by SDS-PAGE and immunoblotted against Cox-IV and GAPDH. C. WT NSC34 cells treated as in B were lysed and proteins were resolved by SDS-PAGE. Immunoblots against Bcl-2 and α-tubulin were completed. Densitometry was completed for Bcl-2 and normalized to α-tubulin. Data are represented as a percent of the Con Bcl-2 expression. *p<0.05 versus Con via an unpaired student’s T-test; n=3, error bar indicate standard error.
4.4.9 The siRNA mediated knockdown of Bcl-2 specifically decreases mitochondrial GSH and re-sensitizes OGC overexpressing NSC34 cells to oxidative stress.

We have previously shown that inhibition of OGC-dependent GSH transport prevents the ability of Bcl-2 overexpression to protect CHO cells from H$_2$O$_2$-induced oxidative stress (Wilkins et al., 2012). Therefore, we hypothesized that Bcl-2 induction may be reciprocally necessary for OGC-dependent protection of NSC34 cells from oxidative stress. To determine if the increased expression of Bcl-2 was instrumental in rendering the OGC stable cell lines resistant to oxidative stress, we used a SMARTPOOL® siRNA to specifically knockdown Bcl-2 in the stable OGC 6 cell line. OGC 6 cells were transfected with either non-target or Bcl-2 siRNA for 48 h and Bcl-2 protein levels were examined. As shown in Figure 4.9.A, the Bcl-2 siRNA markedly reduced Bcl-2 protein levels compared to the non-target control (an average of 47.3% +/- 8.1 % reduction in Bcl-2 protein levels, mean +/-SEM, n=5). Next, we examined the effects of reduced Bcl-2 levels on mitochondrial GSH content. Knockdown of Bcl-2 protein levels induced a specific 35% reduction in mitochondrial GSH content compared to non-target transfected cells (Figure 4.9.B). Cox-IV and GAPDH blots indicate pure cellular fractionations (Figure 4.9.B). Based on the specific reduction in mitochondrial GSH with siRNA-mediated knockdown of Bcl-2 protein levels, we hypothesized that these cells would be re-sensitized to oxidative stress. Indeed, OGC 6 cells transfected with siRNA against Bcl-2 displayed a statistically significant reduction in cell viability with both concentrations of H$_2$O$_2$, whereas no decrease in cell viability was observed with the non-target control (Figure 4.9.C). Experiments were also completed comparing
the non-target siRNA against a no transfection control and no significant differences in
Bcl-2 protein levels, mitochondrial GSH content, or cell viability were observed (data not shown).
Figure 4.9 The siRNA mediated knockdown of Bcl-2 specifically decreases mitochondrial GSH and re-sensitizes OGC overexpressing NSC34 cells to oxidative stress. A. Stable OGC 6 NSC34 cells were transfected with either non-target or Bcl-2 siRNA at 25 nM for 48 h. Cells were lysed, proteins were resolved by SDS-PAGE and immunoblotted against Bcl-2 and β3-tubulin. Densitometry of Bcl-2 normalized to β3 tubulin showed a 43.3% +/- 8.1% (mean +/- SEM, n=5) reduction in Bcl-2 levels in siRNA Bcl-2 transfected cells compared to non-target transfection. B. Cells transfected as in A, then fractionated by differential centrifugation and total GSH content was measured using a colorimetric DTNB assay. Data are represented as a percent of the Non-target control. *p<0.05 compared to non-target via an unpaired student’s T-test, n=6, error bars indicate standard error. C. Cells were transfected as in A, treated with vehicle control (Con); 500 μM or 1 mM H₂O₂. Cell viability was assed via an MTT assay. *p<0.05 versus Con siRNA; **, p<0.01 versus con siRNA; NS, not significant via a one-way ANOVA with posthoc Tukey’s test. n=5, error bars indicate standard error.
4.4.10 Bcl-2 and OGC co-immunoprecipitate from rat brain lysates in a GSH-dependent manner

As stated previously, Bcl-2 and OGC have been demonstrated to be interacting partners. However, past studies have used recombinant and protein overexpression systems to show the association between these two proteins (Wilkins et al., 2012). Therefore, we aimed to determine if the Bcl-2 and OGC interaction could be observed in a system with endogenous levels of protein expression. Here, we used whole rat brain lysates in the presence of high (10 mM) GSH to perform co-immunoprecipitation reactions. As shown in Figure 4.10.A, Bcl-2 co-immunoprecipitated with OGC and OGC co-immunoprecipitated with Bcl-2 in a GSH-dependent manner. The co-immunoprecipitation did not occur without the addition of high GSH (Figure 4.10.B). Finally, an IgG immunoprecipitation is shown as a control for non-specific binding (Figure 4.10.A).
Figure 4.10 Bcl-2 and OGC co-immunoprecipitate from rat brain lysates in a GSH-dependent manner A. Whole rat brain was homogenized in 0.1% Triton X-100 in lysis buffer with protease inhibitors and 10 mM GSH-MEE. Immunoprecipitation reactions were completed using either an IgG, Bcl-2, or OGC antibody. Samples were resolved by SDS-PAGE and immunoblotted against Bcl-2 and OGC. Tot=total lysate. B. Samples were prepared as in A except without GSH-MEE. Immunoprecipitation reactions were completed using either an IgG or OGC antibody. Samples were resolved by SDS-PAGE and immunoblotted against Bcl-2 and OGC. Tot=total lysate.
4.5 Discussion

Previous studies have shown that the overexpression of mitochondrial GSH transporters (OGC and DIC) renders kidney and liver derived cell lines resistant to apoptotic stimuli (Xu et al., 2006; Zhong et al., 2008). However, very limited information is available on the role of these mitochondrial GSH transporters in neuronal systems. Here, we generated stable V5-tagged OGC cell lines using immortalized mouse NSC34 motor neuron-like cells. NSC34 motor neuron-like cells afforded ease of transfection, however, because NSC34 cells are an immortalized cell line, they only approximate the phenotypic characteristics of motor neurons. NSC34 cells differ from primary motor neurons because they lack synaptic transmission; however they do share some similarities with motor neurons such as, sensitivity to insults that block voltage-gated ion channels and axonal transmission (Durham et al., 1993). Furthermore, the stable NSC34 OGC cell lines appeared to retain their neuronal phenotype, as shown through the continued expression of neuron-specific β3 tubulin, despite showing enhanced proliferation compared to parental NSC34 cells. Stable overexpression of OGC in the NSC34 cells led to a significant and specific increase in mitochondrial GSH levels and a corresponding increase in resistance of these cells to oxidative stress. These data suggest an interesting question of whether OGC might play a role in mitochondrial GSH transport within primary motor neurons. Indeed, OGC is abundantly expressed in mouse spinal cord (Wilkins and Linseman, unpublished data). Thus, further studies are warranted in primary motor neurons, especially given the profound resistance observed with enhanced
mitochondrial GSH transport against both oxidative and nitrosative stress, major
pathogenic factors in neurodegenerative motor diseases like ALS.

While it may be anticipated that overexpression of a mitochondrial GSH
transporter would lead to an increase in mitochondrial GSH and enhanced neuronal cell
resistance to oxidative and nitrosative stress, it is quite novel that stable OGC cells also
displayed a marked increase in Bcl-2 protein levels. Furthermore, the upregulation of
Bcl-2 could be recapitulated by chronic exposure of parental NSC34 cells to a cell-
permeable form of GSH, which similarly produced a specific increase in mitochondrial
GSH levels. Our data are consistent with previous studies which showed that modulation
of total cellular GSH levels results in the alteration of Bcl-2 protein levels (Ho et al.,
1997; Celli et al., 1998). However, these prior studies only examined the relationship
between manipulating total cellular GSH content and Bcl-2 protein expression. To our
knowledge, this is the first study to demonstrate that Bcl-2 protein expression is explicitly
regulated by mitochondrial GSH levels.

Several mechanisms could lead to an increase in Bcl-2 protein levels, including
decreased proteasome-dependent degradation, de-repression of Bcl-2 transcription or
translation (such as through microRNAs), or upregulation of Bcl-2 transcription. Celli et
al. (1998) showed that Bcl-2 protein levels were down-regulated in response to depletion
of total cellular GSH levels in a liver cell line due to increased Bcl-2 protein degradation
by the proteasome. MicroRNA involvement has also been implicated in the regulation of
Bcl-2 expression. Two microRNAs, miR-15 and miR-16, have been shown to down-
regulate Bcl-2 expression, a process that is deficient in chronic lymphocytic leukemia
lymphocytes (Cimmino et al., 2005). Whether microRNAs are relevant to the upregulation of Bcl-2, demonstrated here in our model is unknown, however it is interesting to note that chronic lymphocytic leukemia lymphocytes have been shown to have an approximate two-fold increase in GSH, a common observation in most cancerous cells which overexpress Bcl-2 (Ferraris et al., 1994).

While the above mechanisms may contribute to increased Bcl-2 protein levels in response to changes in total cellular GSH, mitochondrial redox alterations specifically, have been shown to modulate protein kinase A (PKA) and downstream cyclic AMP response element binding protein (CREB) transcriptional activities (Lee et al., 2005; Ryu et al. 2005). Importantly, CREB has been shown to contribute significantly to Bcl-2 transcriptional regulation and is known as a redox sensitive transcription factor (Wilson et al., 1996). PKA is activated through an auto-phosphorylation event induced by cyclic AMP (cAMP), and a discrete pool of PKA appears to localize to the outer mitochondrial membrane where it is associated with A kinase anchoring protein (AKAP121) (Affaitati et al., 2003; Ginsberg et al., 2003). It has been previously shown that cAMP generated through a mitochondrial soluble adenylyl cyclase which is sensitive to ATP production, allows for the phosphorylation and activation of mitochondrial localized PKA (Acin-Perez et al., 2009). Furthermore, antioxidants such as the iron chelator deferoxamine, can increase the activity of mitochondrial PKA/CREB, while inhibition of PKA prevented the protective action of deferoxamine against GSH depletion (Ryu et al., 2005). Finally, the association of PKA with AKAP121 has been shown to promote the translocation of mRNA of nuclear encoded mitochondrial proteins from the cytosol to the outer
mitochondrial membrane, thus allowing their translation to occur locally at that site (Ginsberg et al., 2003). While, we have not yet elucidated the mechanism allowing for the upregulation of Bcl-2 protein levels in response to chronically elevated mitochondrial GSH, an increase in Bcl-2 transcription through the PKA/CREB axis, specifically at the level of the mitochondria, is most supported by previous studies. Overall, our data indicate a novel mitochondria-to-nucleus redox sensitive signaling mechanism in the regulation of Bcl-2 protein expression.

We have previously shown that Bcl-2 interacts with OGC in a GSH-dependent manner using both recombinant and protein overexpression systems (Wilkins et al., 2012). The Bcl-2 homolog, CED9, was also shown to be an interacting partner for the OGC homolog, MISC-1, in *C. elegans*, suggesting this interaction is evolutionarily conserved (Gallo et al., 2011). Here, we show that Bcl-2 and OGC interact in a GSH-dependent manner in rat brain lysates, thus verifying this interaction within an endogenous expression system. We have previously demonstrated the dependence of the protective effects of Bcl-2 on the transport function of OGC; treatment of CHO cells overexpressing both Bcl-2 and OGC with the OGC inhibitor, phenylsuccinate, prevented the ability of Bcl-2 to protect from oxidative stress (Wilkins et al., 2012). Our current data show that the knockdown of Bcl-2 through siRNA prevents the ability of overexpressed OGC to protect NSC34 cells from oxidative stress. The knockdown of Bcl-2 also led to a significant and specific decrease in mitochondrial GSH levels in the stable OGC overexpressing cell lines. Therefore, we have provided compelling evidence that OGC and Bcl-2 are interacting partners that work in conjunction to modulate
mitochondrial GSH transport and sensitivity to oxidative and nitrosative stress conditions. Moreover, the protection against oxidative stress afforded by Bcl-2 or OGC overexpression are reciprocally dependent upon one another.

Overall, we show that enhancing mitochondrial GSH transport through the overexpression of OGC provides two main cytoprotective mechanisms. First, overexpression of OGC specifically increased mitochondrial GSH content and rendered NSC34 cells resistant to oxidative and nitrosative stress conditions. Second, the specific increase in mitochondrial GSH induced by the overexpression of OGC produced a corresponding increase in Bcl-2 protein levels. These findings are important to the field of neurodegeneration especially, because Bcl-2 expression is often decreased in these diseases. For example, Bcl-2 expression is diminished in spinal cord tissue from both ALS patients and in a mutant SOD1 mouse model of familial ALS (Mu et al., 1996; Vukosavic et al., 1999). Furthermore, it has been shown that the ratio of GSH/GSSG is also decreased in mitochondria isolated from spinal cord of mutant SOD1 mouse models of familial ALS, and we have recently found that total mitochondrial GSH content is significantly decreased in lumbar spinal cord from end-stage G93A mutant SOD1 mice (Ferri et al., 2006; Pesaresi et al., 2011; Wilkins and Linseman, unpublished data). Therefore, modulating mitochondrial GSH transport could prove to be a significant therapeutic target in neurodegenerative diseases like ALS in which Bcl-2 expression is diminished.
4.6 Innovation

Increased expression of Bcl-2 has previously been shown to enhance cellular GSH content. Here, we show that specifically increasing mitochondrial GSH content through OGC-dependent transport reciprocally induces Bcl-2 expression. Moreover, this increased Bcl-2 expression is necessary for the enhanced resistance of neuronal cells to oxidative stress provided by overexpression of OGC. These data suggest a novel redox sensitive mitochondria-to-nucleus signaling mechanism for the regulation of Bcl-2 expression and identifies mitochondrial GSH transport as a new therapeutic target in neurodegenerative diseases in which Bcl-2 expression is diminished.
CHAPTER FIVE: DISCUSSION

Emerging evidence suggests that the discrete pool of mitochondrial GSH is critical for cell survival. Despite this, the role mitochondrial GSH transport contributes in determining cell resistance to apoptosis has not been elucidated, particularly in neurons. Mechanisms of mitochondrial GSH transport within the CNS are not well understood and this includes both the carriers responsible and their regulation. The pro-survival protein, Bcl-2, which acts as a suppressor of pro-apoptotic proteins, also functions in an antioxidant-like mechanism. However, studies indicating a significant correlation between Bcl-2 and cellular GSH trafficking had not been furthered, until recently. Zimmermann et al. described a novel role for Bcl-2 as a GSH binding protein and depicted that inhibition of Bcl-2 led to mitochondrial GSH efflux (Zimmermann et al., 2007). Thus, the major goals of this thesis were to provide insight into the role Bcl-2 may contribute in maintaining the mitochondrial GSH pool, the carriers responsible for mitochondrial GSH transport in neurons and astrocytes, and the significance of mitochondrial GSH transport in neuronal survival.

5.1 Summary of Major Findings

Chapter 2 of this thesis provided evidence for a direct role of Bcl-2 in mitochondrial GSH transport. Inhibition of Bcl-2 led to a specific and significant reduction in mitochondrial GSH levels, induced MOS, prevented the interaction between
Bcl-2 and GSH, and inhibited mitochondrial GSH transport. After these observations, a mechanism in which Bcl-2 contributes to mitochondrial GSH transport was shown through a novel GSH-dependent Bcl-2/OGC interaction. In addition, the observation that Bcl-2 and OGC work in a concerted manner to enhance mitochondrial GSH transport and that Bcl-2 is dependent on the transport function of OGC to protect against oxidative stress, further demonstrates the importance of these proteins in regulating mitochondrial redox status through GSH.

The main goals of Chapters 3 and 4 of this thesis were to examine the importance of mitochondrial GSH transport in neuronal systems. Chapter 3 elucidated that mitochondrial GSH transport mechanisms are variable between primary cerebellar astrocytes and CGNs. In addition, discrete inhibition or molecular knockdown of a single mitochondrial GSH transporter, in CGNs, rendered these primary neurons more susceptible to oxidative and nitrosative stress conditions. Chapter 4 focused on the protective ability of enhanced mitochondrial GSH transport. In order to examine this a stable cell line overexpressing OGC was developed in the motor neuron-like cell, NSC34 (mouse neuroblastoma fusion with mouse motor neuron cultures). The stable OGC cell lines were significantly resistant to oxidative and nitrosative stress, as well as the GSH depleting agent, ethacrynic acid. The major finding in Chapter 4 was the ability of enhanced mitochondrial GSH content to induce an upregulation of Bcl-2 protein levels. Similar to the finding of Chapter 2, the ability of OGC to protect from oxidative stress was dependent on enhanced Bcl-2 expression. Finally, Bcl-2 and OGC were shown to interact endogenously from rat brain lysates in a GSH-dependent manner. The work
completed in this thesis provides substantial evidence for a role of Bcl-2 in mitochondrial GSH transport and the significant importance of mitochondrial GSH transport in determining neuronal viability. However, what contribution does enhancing the bioavailability of GSH, within the context of neurodegenerative diseases, provide in regards to providing an efficacious treatment option? This question and the role of mitochondrial GSH transport within ALS disease pathology are discussed below.

5.2 Enhancing Glutathione synthesis as a therapeutic approach for ALS

It is not surprising that antioxidant therapies have been examined in neurodegenerative diseases, given the role oxidative and nitrosative stress contribute to disease pathogenesis; however all of the various antioxidants attempted (including enhancing GSH synthesis by providing GSH precursors) have failed in clinical trials especially for ALS. The efficacy of modulating GSH levels within the context of ALS has been previously studied through the use of GSH precursors, such as N-acetylcysteine (NAC) and N-acetylmethionine (NAM). In the G93A mutant SOD1 mouse model of ALS, NAC significantly delayed disease progression and prolonged survival (Andreassen et al., 2000). However, in clinical trials NAC failed to show any discernible effect on disease progression or survival in a randomized, double blind, placebo controlled study (Louwerse et al., 1995). In a separate clinical trial, a combination therapy which included NAC and NAM (among other antioxidants), reported no significant effect on survival for ALS patients (Vyth et al., 1996). To further elucidate the efficacy of GSH precursors as a therapeutic intervention for ALS, a whey protein supplement which has a high content of double bonded cysteine (cystine), known as Immunocal®, was tested in the G93A
mutant SOD1 mouse model. Immunocal® preserved GSH levels and the GSH/GSSG ratio in blood and lumbar spinal cord from end-stage G93A mutant SOD1 mice, compared to non-treated mutant mice (Figure 5.1). Immunocal® treatment led to a significant delay in disease onset and slowed the rate of motor decline, as shown through grip strength measurements (Figure 5.2). However, no effect was observed on survival with Immunocal® in the G93A mutant SOD1 mouse model (Figure 5.2). Overall, these data indicate two possible hypotheses, first being that antioxidant therapy particularly at the level of increasing GSH synthesis is not an efficacious ALS therapy. The second hypothesis, is that while providing precursors for GSH synthesis appears to delay disease onset in animal models of ALS (this is not an effect that could be observed in human patients), it is unable to provide an effect on disease survival because the available GSH pool is unable to be transported into mitochondria. As discussed earlier, mitochondrial GSH has been shown in diverse models to be the most critical reservoir of this antioxidant for cell survival (Meredith and Reed, 1982; Meredith and Reed, 1983; Wüllner et al., 1999; Muyderman et al., 2007). Indeed, upon examination of mitochondrial GSH levels in lumbar spinal cord of end-stage G93A mutant SOD1 mice, it was found that Immunocal® failed to preserve this critical pool of GSH (Figure 5.3). These findings suggest the interesting possibility that the failure of GSH synthesis precursors within ALS clinical trials may stem from a failure of the newly synthesized GSH to be efficiently transported into mitochondria.
Figure 5.1 HPLC-EC detection reveals whole blood and lumbar spinal cord GSH is depleted in the hSOD1G93A mouse model of ALS and is preserved at NonTG levels in hSOD1G93A mice receiving Immunocal®. A. Mean GSH concentrations from the whole blood of end stage trial animals, ** p<.01, * p<.05, n=13. B. GSH/GSSG ratios from the whole blood of end stage trial animals, ** p<.01, n=13. C. Mean GSH concentrations from the lumbar spinal cord of end stage trial animals. * p<.05, n=17. D. GSH/GSSG ratios from the lumbar spinal cord of end stage trial animals. * p<.05, n=17. I acknowledge Erika Ross for assistance in generating the data for this figure.
Figure 5.2 Immunocal® delays clinical onset and diminishes the rate of decline in grip strength in the hSOD1G93A mouse model of ALS. A. Median survival is not significantly different between hSOD1G93A mice receiving Immunocal® ad libitum beginning at 60 days of age and untreated mutant mice (n=13). B. hSOD1G93A mice receiving Immunocal® ad libitum beginning at 60 days of age displayed a delay in disease onset and clinical decline compared to untreated mutant mice (n=13). Onset curves are significantly different (p<.001) determined by Gehan-Breslow-Wilcoxon Test. C. PaGE hanging wire test represented as (mean ± SEM) latency to fall at indicated ages (n=13). *** indicates p<.001 compared to NonTG, ** indicates p<.01 compared to NonTG, † indicates p<.05 compared to hSOD1G93A mice (one-way ANOVA with a post-hoc Tukey’s test). I acknowledge Erika Ross for assistance in generating the data for this figure.
Figure 5.3 Immunocal® fails to preserve mitochondrial GSH levels from lumbar spinal cord of end-stage G93A SOD1 mice. Mitochondria were isolated from lumbar spinal cord using a Percoll gradient and differential centrifugation. Total GSH was measured using a DTNB colorimetric assay. All measurements of total GSH are normalized to protein and represented as nmols/mg. *p<0.05, **p<0.01, NS=not significant via a One-way ANOVA with post hoc Tukey’s test, n=4, error bars indicated SEM.
Mitochondrial GSH levels in this disease model were further examined at disease onset (~90 days) and end-stage (~120 days) time points in lumbar spinal cord and cortex. Mitochondrial GSH did not show a significant reduction in either lumbar spinal cord or cortex at disease onset in the G93A mutant SOD1 mouse model (Figure 5.4). However, end-stage animals displayed a significant decrease in mitochondrial GSH in lumbar spinal cord. This effect was not significant in mitochondria isolated from cortex, but did display a trend towards significant depletion (Figure 5.4). These data are consistent with prior studies showing that G93A mutant SOD1, both \emph{in vitro} (overexpression in NSC34 cells) and \emph{in vivo} (mouse model) causes a disruption in mitochondrial GSH/GSSG ratios (Ferri et al., 2006; Pesaresi et al., 2011). Next, the ability of mitochondria from this mouse model to transport GSH was assessed. While mitochondria isolated from both wild-type (NonTg) and G93A mutant SOD1 lumbar spinal cord displayed a large increase in GSH content during the incubation period, there was an \~50% reduction in the ability to take up GSH in mitochondria isolated from G93A mutant SOD mice (Figure 5.4). The effect observed was specific to isolated mitochondria from lumbar spinal cord of G93A mutant SOD1 mice, as mitochondria isolated from mutant cortex did not show a deficit in mitochondrial GSH uptake compared to NonTg controls (Figure 5.4). Overall, these data strongly support the notion that mitochondrial GSH transport is compromised in the G93A mutant SOD1 mouse model of ALS.
Figure 5.4 Mitochondrial GSH levels and transport in the G93A mutant SOD1 mouse model

A. Mitochondria were isolated from WT (NonTg) G93A mutant SOD1 (hSOD1^{G93A}) mice at onset (~90 days old) and end-stage (~120 days old) from lumbar spinal cord and whole cortex as described in Figure 5.3. Total GSH was measured using a DTNB colorimetric assay. All measurements were normalized to protein and represented as a percent of NonTg. ***p<0.001, NS=not significant via an unpaired student’s T-test, n=4, error bars indicated SEM.

B. Mitochondria were isolated from lumbar spinal cord and cortex of end-stage (~120 days old) G93A mutant SOD1 (hSOD1^{G93A}) and age-matched WT (NonTg) mice as described in Figure 5.3. Isolated mitochondria were incubated with 2 mM GSH at 37 °C, 300 rpm for 4 h. After which, mitochondria were washed 3x and total GSH was measured using a DTNB colorimetric assay. All measurements were normalized to protein and represented as μmoles/mg of GSH loaded per hour. *p<0.05, NS=not significant, via a paired student’s T-test, n=7, error bars indicated SEM.
5.3 Mechanisms of decreased mitochondrial glutathione transport in ALS

There are several mechanisms by which mitochondrial GSH transport could be compromised within the context of ALS disease pathology. First, Bcl-2 expression and function are compromised in ALS and as this thesis has depicted, Bcl-2 contributes a significant role in mitochondrial GSH transport. Second, the expression levels of other mitochondrial GSH carriers could be down-regulated in the lumbar spinal cord, such as DIC and OGC. Finally, post-translational modifications that occur with increased oxidative and nitrosative stress (such as S-nitrosylation, thiol oxidation, or carbonylation), may compromise function of Bcl-2 or the previously identified mitochondrial GSH transporters. All of these hypotheses are discussed below.

5.3.1 Aberrant changes in Bcl-2 function and expression as a mechanism of decreased mitochondrial glutathione transport in ALS

The compromise of Bcl-2 function and expression in ALS could lead to a profound impact on the ability of mitochondria to transport GSH. In the G93A mutant SOD1 mouse model of ALS, Bcl-2 adopts a “toxic” conformation induced through its interaction with mutant SOD1 (Pasinelli et al., 2004; Pedrini et al., 2010). This “toxic” conformation exposes the pro-apoptotic BH3-domain of Bcl-2, potentially transforming it into a death inducing protein (Pedrini et al., 2010). Bcl-2 also interacts with WT SOD1 however; Bcl-2 associates more strongly with high molecular weight aggregates of mutant SOD1, particularly at mitochondria (Pasinelli et al., 2004). WT SOD1 does not induce the same “toxic” conformational change in Bcl-2 as that observed with mutant
SOD1 (both G93A and G37R) (Pedrini et al., 2010). Moreover, the interaction between Bcl-2 and high molecular weight aggregates of mutant SOD1 was specific to lumbar spinal cord in both mice and ALS patients (harboring A4V SOD1 mutation specifically), as the same observation was not found in liver (Pasinelli et al., 2004). It was shown in Chapter 2 of this thesis, that a BH3-mimetic (HA14-1) prevented the interaction between Bcl-2 and GSH, while also specifically depleting mitochondrial GSH. Combined with the data presented above showing that G93A mutant SOD1 mice have compromised mitochondrial GSH transport, the ability of G93A mutant SOD1 to disrupt the interaction between GSH and Bcl-2 was investigated. As shown in Figure 5.5, upon incubation of recombinant Bcl-2 with recombinant mutant G93A SOD1, GSH-agarose beads failed to pull Bcl-2 out of solution, while incubation with WT SOD1 had no effect on this Bcl-2/GSH interaction. Incubation of isolated rat brain mitochondria with G93A mutant SOD1 also led to an inhibition of GSH uptake when compared to WT SOD1 (Figure 5.5). While more experiments are necessary, the aberrant interaction between mutant SOD1 and Bcl-2 could contribute to deficits of mitochondrial GSH transport within this ALS mouse model.

In addition to altered function of Bcl-2 through an interaction with mutant SOD1, previous studies have shown that Bcl-2 levels are decreased in patients and mouse models of ALS. Decreased Bcl-2 levels were not observed pre-symptomatically, but were apparent post-symptomatically in the G93A mutant SOD1 mouse model and this effect was specific to spinal cord (Vukosavic et al., 1999). In addition, Bcl-2 mRNA levels are decreased in lumbar spinal cord tissues isolated post-mortem from sporadic ALS patients.
(Mu et al., 1996). Therefore, while inhibition of Bcl-2 function may contribute to decreased mitochondrial GSH transport, diminished Bcl-2 expression levels may also play a role in this deficit.
Figure 5.5 Effects of G93A mutant SOD1 on the ability of Bcl-2 to bind GSH and mitochondrial GSH transport

A. Recombinant Bcl-2 was incubated with GSH-agarose beads (GSHAg), Agarose beads (Ag), GSH-agarose beads with 25 ng of recombinant G93A mutant SOD1 (25ng G93A SOD1) all in the presence of 1 mM DTT in lysis buffer, for 1 h at 4°C, mixing by inversion. Samples were spun down and washed 3x (with lysis buffer) and proteins were resolved by SDS-PAGE. Immunoblots were completed against Bcl-2, n=2. B. Samples were prepared as in A, except using 25 ng WT SOD1 (25 ng WT SOD1), n=2. C. Mitochondria were isolated from rat brain using a Percoll gradient and incubated for 4 h at 37°C, 300 rpm in the presence of either 25 ng WT SOD1 (25 ng WT) or 25 ng G93A SOD1 (25 ng G93A) and 2 mM GSH. Mitochondria samples were washed 3x and total GSH was measured using a DTNB colorimetric assay. Data are indicated as percent of Control (Con), n=2.
5.3.2 Alterations in protein levels of mitochondrial glutathione transporters as a mechanism of decreased mitochondrial glutathione transport in ALS

While alterations in Bcl-2 function and expression are a likely contributor to decreased mitochondrial GSH transport, decreased expression of mitochondrial GSH carriers could also lead to similar deficits. A previous study compared mitochondrial protein composition from lumbar spinal cord, between WT and G93A mutant SOD1 mice, and found a subset of proteins were either upregulated or down-regulated in the G93A mutant SOD1 mouse model (Li et al., 2010). Two carriers from the SLC family (Slc25a4 and Slc25a5; ADP/ATP translocase ANT 1 and 2) were down-regulated, however the study did not find any previously identified mitochondrial GSH carriers to have altered expression levels (Li et al., 2010). This study is in agreement with immunoblotting completed from lumbar spinal cord in the G93A mutant SOD1 mouse model, examining OGC and DIC protein levels, which did not show a difference compared to WT mice in expression levels (data not shown). While it appears a down-regulation in mitochondrial GSH transporter protein levels has not yet been observed, two caveats need to be discussed. First, it is impossible to distinguish between cell types with immunoblotting, therefore, immunohistochemical studies are needed to determine if mitochondrial GSH transporter protein level alterations are possibly cell type specific. However, given the data showing mitochondrial GSH and transport are decreased from mitochondria isolated from whole lumbar spinal cord, this may not be the case. Finally, the specific carriers responsible for mitochondrial GSH transport in lumbar spinal cord motor neurons versus astrocytes needs to be distinguished so those specific proteins can
be examined. However, current observations do not support a role for decreased protein expression of known mitochondrial GSH carriers in mitochondrial GSH transport deficits within the G93A mutant SOD1 mouse model of ALS.

5.3.3 Protein modifications of mitochondrial glutathione transporters or Bcl-2, as a mechanism of decreased mitochondrial glutathione transport in ALS

Protein function can be significantly altered by modifications of key residues, particularly in environments of enhanced oxidative and nitrosative stress, such as that observed in ALS. Therefore, the last hypothesis for the mechanism of reduced mitochondrial GSH transport within the G93A mutant SOD1 mouse is oxidative or nitrosative modification of amino acid residues within mitochondrial GSH transporters or Bcl-2, leading to disrupted function of these proteins.

Cysteine residues are often cited as critical for protein function and this is based on their role in active sites as well as intra- and inter-molecular disulfide bridge formation. In particular, OGC contains three cysteine residues (C184, C221, and C224), two of which form an intramolecular disulfide bridge (C221 and C224). Mutagenesis of C221 and C224 into serine residues significantly reduces the transport ability of OGC (Xu et al., 2006). In comparison, DIC has five cysteine residues, and when mutated to serine or alanine, no effect on DIC transport function was observed (Lash, 2006). However, isolated recombinant DIC is very susceptible to thiol oxidation, which leads to its subsequent inactivation. Bcl-2 contains two cysteine residues and mutagenesis of both leads to a reduced capacity in its anti-apoptotic function while retaining its ability to interact with the pro-apoptotic protein, Bax (Maser et al., 2000). Evidence of alterations
in cysteine residues has been found in the plasma of ALS patients, which have a
significant reduction in free thiol content compared to control subjects (Baillet et al.,
2010). Therefore, oxidative or nitrosative modifications to key cysteine residues within
OGC, DIC, or Bcl-2 may lead to decreased mitochondrial GSH transport as discussed
below.

There are several oxidative modifications of protein thiols which can affect
function, including thiyl radicals (-RS\(^{-}\)), sulfenate (-SO\(^{-}\)), sulfinate (-SO\(_2\)^{-}\), and sulfonate
(SO\(_3\)^{-}\) formation. Thiyl radical formation is extremely transient, resulting in disulfide
bond formation; sulfenic acid is a reversible modification, while sulfinate and sulfonate
are thought to be irreversible within mammalian systems (Jones, 2008). It has been
hypothesized that thiol containing proteins involved in redox signaling are susceptible to
oxidative modifications, especially by non-radical oxidants (i.e. H\(_2\)O\(_2\)) (Jones, 2008). In
particular, sulfenic acid formation through the oxidation of thiols has been proposed to
alter the biological function of proteins through the modification of allosteric cysteines
(Jones, 2008). This may be particularly relevant in regards to the hypothesis presented in
Chapter 2 of this thesis, where the interaction between Bcl-2 and GSH, possibly induces a
conformational change (allosteric) in Bcl-2 which increases its affinity for OGC.
Furthermore, the formation of sulfenic acid through the oxidation of the cysteine residues
of Bcl-2, by H\(_2\)O\(_2\), has been shown to induce a down-regulation of Bcl-2 protein levels
and apoptosis (Luanpitpong et al., 2013). However, no evidence for sulfenic acid
modification has been observed currently for the mitochondrial GSH transporters, OGC
and DIC.
Nitric oxide-induced modifications to cysteine residues, or S-nitrosylation, can also induce an inhibitory effect on protein function. While no evidence has yet been observed regarding S-nitrosylation of DIC, a previous study identified C184 of OGC as an S-nitrosylation site within the myocardium (Kohr et al., 2011). C184 is not a part of the intramolecular disulfide bridge of OGC, but is hypothesized to act as a redox sensor for OGC function (Lash, 2006). Bcl-2 has been shown to be s-nitrosylated; however this modification prevented the ubiquitination and subsequent degradation of Bcl-2; thus was a protective mechanism from apoptosis (Azad et al., 2006). Overall, the literature supports different consequences of s-nitrosylation for Bcl-2 and OGC functions.

While there are many other forms of oxidative and nitrosative modifications to amino acids, protein carbonylation (oxidative) and 3-nitrotyrosine (3-NT; induced by peroxynitrite) modifications are increased in ALS patients and the G93A mutant SOD1 mouse model (Beal et al., 1997; Ferrante et al., 1997; Siciliano et al., 2007; Andrus et al., 1998). In addition, a recent study elucidated that depletion of GSH induced a significant increase in protein carbonylation, an effect that was required for apoptosis, in a neuron-like PC12 cell line (Dasgupta et al., 2012). While it is not surprising that no evidence has been published regarding carbonylation of OGC and DIC thus far, Bcl-2 has previously been shown to be carbonylated prior to apoptotic cell death in the insulin-secreting RINm5F cell line in response to nitric oxide (Cahuana et al., 2004). Currently, no observations are published regarding 3-NT modifications of Bcl-2, OGC, or DIC.

Overall, the possible effects of oxidative or nitrosative modifications to Bcl-2 and previously identified mitochondrial GSH transporters warrants investigation in the G93A
mutant SOD1 mouse model of ALS. Based on current literature, evidence depicts that oxidative modifications can inhibit the ability of Bcl-2 to prevent apoptosis; however no current literature has examined the effect of oxidative modifications on OGC or DIC function. Furthermore, the effects that these potential oxidative modifications induce on the mitochondrial GSH transport functions of these proteins necessitate examination.

5.4 The role of decreased mitochondrial glutathione transport in the mechanism of ALS disease pathology

While this thesis has provided evidence of decreased mitochondrial GSH transport in the G93A mutant SOD1 mouse model of ALS, the relevance of this finding to disease mechanism and pathology requires discussion. Reactive gliosis imparts a large role on ALS disease onset and progression, as shown through the effect of targeted overexpression of Nrf2 (Nuclear factor (erythroid-derived 2)-like 2); a transcription factor which upregulates antioxidant defense proteins; such as GSH synthesis enzymes) in astrocytes of mutant SOD1 mouse models. Vargas and colleagues showed that overexpression of Nrf2 within astrocytes increased GSH content and secretion. Astrocytes overexpressing Nrf2 protected co-cultured motor neurons from G93A mutant SOD1 toxicity, an effect that was dependent on GSH secretion (Vargas et al., 2008). In vivo, targeted Nrf2 overexpression in astrocytes significantly delayed disease onset and extended survival in two distinct mutant SOD1 mouse models (Vargas et al., 2008). Finally, exposure of astrocytes to peroxynitrite induces a neuroinflammatory phenotype, apoptosis of co-cultured motor neurons, and an increase in nitrotyrosine levels (Cassina et al., 2002). Therefore, the oxidative balance in astrocytes appears to greatly influence
the induction of a neuroinflammatory phenotype and more importantly, disease
progression in mutant SOD1 mouse models.

In additional studies, the role of GSH in the ability of astrocytes to protect co-
cultured neurons has been extensively studied. Total cellular GSH depletion within
astrocytes prevented the protection of co-cultured neurons from nitrosative stress and
\( \text{H}_2\text{O}_2 \) (Drukarch et al., 1997; Chen et al., 2001). Furthermore, astrocytes depleted of
GSH and co-cultured with neurons, induced a loss in neuronal survival and dopamine
transport (Drukarch et al., 1997). GSH depletion of astrocytes has also been shown to
affect neuronal mitochondrial function. Specifically, GSH depleted astrocytes secreted
significantly less GSH and induced a decrease in co-cultured neuronal complex II, III,
and IV activities (Gegg et al., 2005). Finally, studies have elucidated that neurons are
more sensitive to GSH depletion when compared to astrocytes (Byrd et al., 2004).
Overall, the GSH status of both neurons and astrocytes greatly influences cell survival
and the susceptibility to oxidative and nitrosative stress \textit{in vitro}.

A more recent study deciphered that astrocytes isolated from the G93A mutant
SOD1 mouse model induce cell death of WT co-cultured motor neurons(Cassina et al.,
2008). This effect could be recapitulated through the inhibition of the electron transport
chain within WT astrocytes (Cassina et al., 2008). More striking, is the result depicting
that treatment of G93A mutant SOD1 astrocytes with mitochondrial targeted antioxidants
prevents the death of co-cultured motor neurons (Cassina et al., 2008). These are the first
observations showing that mitochondrial oxidative stress within astrocytes is required for
the induction of cell death in co-cultured neurons.
In addition to studies showing that MOS and GSH play an important role in the ability of astrocytes to either be helpful or harmful neighbors to motor neurons, this thesis has found several novel observations relating to the role of mitochondrial GSH transport in neuronal viability. First, Bcl-2 and OGC work in a concerted manner in mitochondrial GSH transport and subsequent inhibition of apoptosis. Second, discrete inhibition of a single mitochondrial GSH transporter within neurons, is sufficient to increase vulnerability to oxidative and nitrosative stress. Third, mitochondrial GSH has a potential role in the regulation of Bcl-2 protein levels, possibly through a signaling mechanism to the nucleus. Finally, while these data were not shown within my thesis, mitochondrial GSH also appears to have a function in regulating mitochondrial dynamics (through enhanced mitochondrial fission and/or biogenesis). Therefore, deficits in mitochondrial GSH transport have the potential to affect many cellular processes either directly or indirectly.

The observations presented within this thesis and discussed above have led to the following hypothesis. Underlying oxidative and nitrosative stress, within astrocytes, leads to deficits in mitochondrial GSH transport (this could be a consequence of any one of the factors discussed above). The underlying oxidative stress is postulated to be a result from either environmental exposures, genetic abnormalities, or both. Overall these events culminate through the induction of a reactive gliosis phenotype, a decrease in GSH secretion and an increase in the release of NO and ROS from the reactive astrocytes. These effects then induce an elevation in the underlying oxidative stress within motor neurons, increasing MOS and dysfunction, as mitochondrial GSH levels plummet.
Decreased mitochondrial GSH levels (due to inhibited transport and increased oxidative stress) will induce a reduction in Bcl-2 protein levels, increased production of ROS and altered cell signaling pathways. These events culminate in motor neuron axon retraction and degeneration, as depicted in Figure 5.6.

In conclusion, the pathogenic mechanisms of ALS are complicated, but deficits in mitochondrial GSH transport, whether a cause or effect in the cascade of disease progression, offers a unifying hypothesis between neuronal and astrocyte interactions. While further evidence is necessary in order to determine more specific clinical trial targets, I hypothesize that targeting mitochondrial GSH transport as a therapy in ALS may provide efficacious treatment options for this devastating neurodegenerative disease.
Figure 5.6 Working model regarding the role decreased mitochondrial GSH transport may contribute to ALS disease pathogenesis. Increased oxidative and nitrosative stress, within astrocytes, leads to deficits in mitochondrial GSH transport, induction of a reactive gliosis phenotype, decreased GSH secretion and increased release of NO and ROS. The orange arrow represents the induction of effects within motor neurons as a result of reactive gliosis onset within astrocytes. Where, an elevation in the underlying oxidative stress within motor neurons can induce a deficit in mitochondrial GSH transport, altered cell signaling pathways, axon retraction and motor neuron degeneration. I would like to acknowledge Aimee Winter for drawing the astrocyte and motor neuron.


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APPENDIX A: ABBREVIATIONS

[3H]GSH; tritiated glutathione
32D; mouse immortalized myeloblast-like cell line
3-NT; 3-nitrotyrosine
5K; 5mM potassium
8-OHdG; 8-hydroxydeoxyguanosine
A4V mutant SOD1; alanine mutated to valine at amino acid position 4 in Cu/Zn superoxide dismutase
ABT-737; BH3 mimetic 4-{4′-(4′-Chlorobiphenyl-2-yl)methyl}piperazin-1-yl}-N-[(4-\{(1R)-3-(dimethylamino)-1-[(phenylsulfanyl)methyl]propyl\}amino)-3-nitrophenyl]sulfonyl]benzamide
ADP; adenine diphosphates
AIF; apoptosis inducing factor
AKAP121; A kinase anchoring protein
ALS; amyotrophic lateral scelorosis
AMPA; 2-amino-3-(3-hydroxy-5-methyl-isoxazol-4-yl)propanoic acid
ANT; Slc25a5 or Slc25a4 adenine nucleotide transporter
AP-1; activator protein 1 transcription factor
AP-2; activator protein 2 transcription factor
ARE/EpRE; antioxidant/electrophile response element
ATP; adenine triphosphate
Bcl-2; B cell lymphoma protein 2
BH; Bcl-2 homology domain
BM; butylmalonate
BSO; buthionine sulfoxide
Ca^{2+}; calcium
CAD; caspase dependent DNase
Cas; caspase
CGN; cerebellar granule neuron
CGN; cerebellar granule neurons
CHO; chinese hamster ovarian cell
CNS; central nervous system
Cox-IV; cytochrome c oxidase or complex IV
CREB; cyclic AMP response element binding protein
CSF; cerebral spinal fluid
CSM14.1; immortalized mesencephalic progenitor cell line
Cyt c; cytochrome c
DIC; Slc25a10 dicarboxylate carrier
DISC; death inducing signaling complex
DMSO; dimethylsulfoxide
DNA; deoxyribonucleic acid
DSS; disuccinimidyl suberate
DTNB; 5′-dithiobis(2-nitrobenzoic acid)
DTT; dithiothreitol
E2F1; E2F transcription factor 1
E2F4; E2F transcription factor 4
EA; ethacrynic acid
EAAT- excitatory amino acid transporter
ER; endoplasmic reticulum
ETC; electron transport chain
FasL; Fas ligand
FDC-P1; mouse bone marrow lymphoblast cell line
FL5.12; mouse pro-B-cell lymphoid cell line
G37R mutant SOD1; glycine mutated to arginine at amino acid position 37 in Cu/Zn superoxide dismutase
G6PD; glucose-6-phosphate dehydrogenase
G93A mutant SOD1; glycine mutated to alanine at amino acid position 93 in Cu/Zn superoxide dismutase
GAPDH; glyceraldehyde 3-phosphate dehydrogenase
GCL- γ-glutamylcysteine ligase
GCLCL- γ-glutamylcysteine ligase catalytic subunit
GCLM- γ-glutamylcysteine ligase modulatory subunit
GPx; glutathione peroxidase
GR; glutathione reductase
GS; glutathione synthetase
GSH; glutathione
GSH-MEE; glutathione monoethylester
GSNO; s-nitrosoglutathione
GSSG; glutathione disulfide
GST; glutathione S transferase
GT1-7; mouse immortalized gonadotropin-releasing hormone neurons
H2O; water
H2O2; hydrogen peroxide
H4IIE; rat hepatoma cell line
HA14-1; 2-Amino-6-bromo-α-cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester
HeLa; human epitheloid cervix carcinoma
HL-60; Human promyelocytic leukemia cell line
HNE; hydroxyl noneal
hSOD1 G93A; human G93A SOD1 transgenic mice
ID2; inhibitor of DNA binding 2
IMM; inner mitochondrial membrane
IP; immunoprecipitation
IP3; Inositol trisphosphate
L929; murine aneuploid fibrosarcoma cell line
LY-ar; mouse lymphoma cells
MCF-7; Michigan cancer foundation 7 human breast adenocarcinoma cell line
mM; millimolar
MOMP; mitochondrial outer membrane permeabilization
MOS; mitochondrial oxidative stress
mPTP; mitochondrial permeability transition pore
mRNA; messenger ribonucleic acid
NAC; n-acetylcytsteine
NAD+; nicotinamide adenine dinucleotide
NADPH/NADP+; nicotinamide adenine dinucleotide phosphate
NAM; n-acetylmethionine
NFκB; nuclear factor kappa-light-chain-enhancer of activated B cells transcription factor
NMDA; N-methyl-D-aspartate
NO; nitric oxide
NonTg-non-transgenic
Nrf1/2; nuclear respiratory factor 1 or 2 also known as nuclear factor (erythriod-derived)-like transcription factors
Nrk52E; rat renal proximal tubular cell line
NSC; neocarzinostatin
NSC34; mouse immortalized motor neuron cell line
NT2/D1; pluripotent human testicular embryonic carcinoma cell line
O2-; superoxide
OGC; Slc25a11 2-oxoglutarate carrier
PBS; phosphate buffered saline
PC12; rat pheochromocytoma adrenal medulla cell line
PD; parkinson’s disease
PhS or PS; phenylsuccinate
PKA; protein kinase A
PM; plasma membrane
Pro-cas; caspase
PS; phenylsuccinate
RNA-ribonucleic acid
ROS; reactive oxygen species
RS-; thyl radical
Sin-1; Amino-3-morpholinyl-1,2,3-oxadiazolium NO donor
Sk-N-MC; human neuronal epithelioma cell line
SLC; solute carrier family
SNAP; S-Nitroso-N-acetyl-DL-penicillamine NO donor
SNP; sodium nitroprusside
SO-; sulfenate
SO2-; sulfinate
SO3-; sulfonate
SOD; superoxide dismutase
SOD1; Cu/Zn superoxide dismutase
SOD2; manganese superoxide dismutase
SP-1; specificity protein 1 transcription factor
STS; starausporine
TCC; Slc25a1 tricarboxylate carrier
TM; transmembrane
TNF; tumor necrosis factor
VDAC; voltage dependent anion channel
WT; wild-type
XAG-; Slc1a1 glutamate transporter
Xc-; Slc7a11 cysteine/glutamate transporter
μM; micromolar
APPENDIX B: PREFACE

Chapter 2 entitled: “Bcl-2 is a novel interacting partner for the 2-oxoglutarate carrier and a key regulator of mitochondrial glutathione” was published in Free Radical Biology and Medicine. 52; 410-19, 2012.

Chapter 3 entitled: “Mitochondrial Glutathione Transport is a Key Determinant of Neuronal Susceptibility to Oxidative and Nitrosative Stress” was published in Journal of Biological Chemistry. 288; 5091-101, 2013.

Chapter 4 entitled: “Stable overexpression of the 2-oxoglutarate carrier enhances neuronal cell resistance to oxidative stress via Bcl-2 dependent mitochondrial GSH transport” is a manuscript in preparation for Antioxidants and Redox Signaling.