Overexpression of Amyloid Precursor Protein Induces Mitochondrial Oxidative Stress and Activates the Intrinsic Apoptosis Pathway

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OVEREXPRESSION OF AMYLOID PRECURSOR PROTEIN INDUCES MITOCHONDRIAL OXIDATIVE STRESS AND ACTIVATES THE INTRINSIC APOPTOSIS PATHWAY

A Thesis
Presented to
The Faculty of Natural Sciences and Mathematics
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In Partial Fulfillment
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Masters of Science

by
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ABSTRACT

Down syndrome (DS) is the most common genetic form of cognitive disability and is caused by trisomy of chromosome 21. Within chromosome 21 is the gene, amyloid precursor protein (APP). Proteolysis of APP into toxic and aggregate-prone, beta-amyloid fragments underlies the pathophysiology of Alzheimer's disease (AD). Individuals with DS develop the neuropathology that can be diagnosed as AD; however, the role of APP overexpression in this comorbidity is presently unclear. Here, we elucidated the mechanism of cell death induced by overexpression of wild type APP. Chinese hamster ovary cells transfected with a DsRed-APP fusion construct displayed caspase-3 activation and nuclear fragmentation indicative of apoptosis. APP-induced apoptosis was blocked by a pan-caspase inhibitor, (BOC), glutathione (GSH), or co-expression of Bcl-2. APP caused depletion of mitochondrial GSH, induced opening of the permeability transition pore, and triggered cytochrome c release. Each of these events was inhibited by GSH but was unaffected by BOC indicating that they were oxidative stress-dependent and upstream of caspases. We conclude that APP overexpression is sufficient to cause mitochondrial oxidative stress and intrinsic apoptosis. We are currently examining if a similar cell death pathway is induced by APP in neuronal cells. Our data are consistent with an increased expression of APP being a likely contributor to neuron death in DS. Thus,
decreasing APP-induced oxidative stress and apoptosis may be beneficial in reducing the comorbid phenotype of DS patients afflicted with AD.
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CHAPTER ONE: INTRODUCTION

1.1 Down Syndrome

Down syndrome (DS) was first described by a British physician, John L. H. Down in 1866, it was first characterized by the mental retardation and unique facial appearances that individuals with down syndrome develop. DS is caused by a partial or full triplication of chromosome 21 (HSA21), the smallest of the human chromosomes. DS occurs in approximately one in every 800 births [1]. When there is a chromosomal triplication in utero in the first and second trimester, DS occurs at a rate of 75% and 50% respectively; unfortunately these trisomic individuals are lost before they make it full term [2]. The hallmarks of DS include intellectual disability, hypotonia, and an abnormally shaped cranium. Individuals with down syndrome present an array of health problems including atrial and ventricular septal defects, dementia (Alzheimer’s), cataracts, leukemia, sleep apnea and hypothyroidism, to name a few. Interestingly, individuals with down syndrome present a decreased risk of breast and lung cancers as well as atherosclerosis [3]. DS arises from a maternal non-disjunction event in meiosis I, which can lead to the triplication of HSA21. Older mothers have an increased risk of producing a child with DS. Full triplication of HSA21 encompasses roughly 95% of DS cases, whereas partial triplication and mosaicism of HSA21 comprise the minority of DS cases [4, 5]. The triplication of HSA21 has caused some genes on that chromosome to be overexpressed by ~50% in many tissues
Currently it is not known how this relatively small increase in transcript levels results in the common features of DS.

Individuals with DS display features of premature aging of which an AD type dementia appears to be a prominent characteristic [6]. These individuals experience increased seizures and by their mid-40s develop the neuropathology of AD characterized by $\beta$-amyloid (A$\beta$) plaques, neurofibrillary tangles consisting of hyperphosphorylated Tau, and basal forebrain cholinergic neuron degeneration [7]. The AD pathology that Individuals with DS develop also leads to a loss of neurons in the hippocampus and cortex, cell populations associated with memory and learning, and information processing respectively. The loss of neurons in these areas is thought to play a significant role in the cognitive disability that individuals with Down syndrome present.

In regards to the relationship between the overexpression of specific genes on HSA21 and the pathology of DS there are two leading hypotheses. The first is the gene dosage hypothesis, which states that due to the presence of an extra HSA21 there is a correspondingly 50% increase in the expression of genes on this chromosome that directly or indirectly alter the timing, pattern or extent of neuronal development [8]. The second hypothesis is the genetic homeostasis hypothesis which states it is the expression of extra genes, regardless of their identity or function, that leads to phenotypic alterations [9]. These two hypotheses aim to explain the abnormal development that is observed in DS individuals. Overexpression of certain genes may play a positive role in
mitigating the pathological consequences of DS such as super oxide dismutase 1 (SOD1), which converts free radicals to less damaging species, or glycinamine ribonucleotide synthase-aminomidazole ribonucleotide synthase-glycinamide formyl transferase (GART), which is involved in catalytic steps of the de novo purine synthesis pathway. Thus, the overproduction of these genes may have positive effects on development by providing successful clearance of free radicals or producing energy via enhanced nucleotide synthesis. GART may have beneficial properties, but increased expression of GART can be harmful due to the increase in purines which can cause gout and in general an increase in uric acid levels. In contrast the overexpression of amyloid precursor protein (APP), which is mutated in some forms of AD which promotes the self aggregation and accumulation of beta-amyloid, and the increased levels of β-site APP-cleaving 2 enzyme (BACE2) (Fig. 1) likely play negative roles in the proper development of DS individuals, especially in the brain.

1.2 Chromosome 21

HSA21 is the smallest of the human chromosomes consisting of the short (p) arm in which the sequence remains unknown and the long (q) arm that was discovered to contain about 225 genes, although many of their functions and specific role in DS remain unclear [10]. The best defined region of HSA21 is known as the “Down syndrome critical region” (DSCR) (Fig. 1). The DSCR segment contains about 33 genes that are conserved in various species, these genes are known to be associated with DS and are suggested to lead to the craniofacial abnormalities, short stature,
joint hyperlaxity, hypotonia and mental retardation that Individuals with down syndrome display [11]. DSCR1 is known as Regulator of Calcineurin (RCAN1), which is highly expressed in brain, heart and skeletal muscle [12]. Also located on HSA21 is the gene, Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase-1A (DYRK1A) that acts to phosphorylate several transcription factors and is known to be involved in neuronal differentiation in the hippocampus [13]. RCAN1 and DYRK1A are both highly studied proteins since they play significant roles in normal cellular function, but there are a host of other genes found on HSA21 that are postulated to also play a large role in the phenotypic pathology of DS such as Ets-2, Cu/Zn SOD1, and APP.
Figure 1: Chromosome 21, Location of the Most Studied DS Genes. The genes located on the p arm of HSA21 are not as well characterized compared to that of the longer q arm. APP, SOD1, DSCR and BACE2 are commonly investigated for their contribution to the neuropathology of DS. [14].

1.2.1 RCAN1

RCAN1 has four splice variants of which RCAN1-1 is the most abundant, producing a protein that is 197 amino acids long and is found primarily and abundantly in the brains of fetuses and adults [15]. RCAN1 co-localizes with synaptophysin suggesting that it is expressed on synaptic vesicles. The overexpression of RCAN1 has been shown to cause aggresomes similar to those observed in DS and AD brains and reduces synaptophysin expression in neural
processes [16]. In DS, abnormal vesicle trafficking precedes the neuronal deposition of Aβ in the brain. Also, there is a decreased recycling of late endosomes as well as an increase of enlarged early endosomes. The negative effect of RCAN1 on synaptophysin implies that overexpression of RCAN1 in DS adversely affects exocytosis, endocytosis and vesicle trafficking, important functions for the normal development and plasticity of a brain.

When neurons overexpress RCAN1 they show a marked increase of mitochondria that exhibit smaller morphologies, an increase of reactive oxygen species (ROS), and decreased ATP production [17]. The increase of ROS in these neurons could play a part in the hippocampal and cortical loss of neurons observed in DS. Poor mitochondrial dynamics and cellular oxidative stress are involved in lethal cellular pathways and will be addressed in detail later in this thesis. Certainly, it is possible that RCAN1 effects such as reducing ATP and increasing production of ROS contributes to the decreased numbers of viable cells in the brains of individuals with DS.

1.2.2 DYRK1A

DYRK1A overexpression has many deleterious effects in DS (Fig. 2). It has been shown to hyperphosphorylate important proteins in the brain, as well as inhibit neuronal precursor proliferation. When the serine-theonine protein kinase, DYRK1A, is overexpressed both APP and Tau show increased levels of phosphorylation [18]. Tau is a microtubule stabilizing protein that is disrupted
from microtubules when hyperphosphorylated. After Tau becomes hyperphosphorylated it promotes self-assembly of axonal tangles. Neuronal tangles and hyperphosphorylated Tau are prominent hallmarks of AD. DYRK1A also leads to AD like pathology by phosphorylating APP on the intracellular domain at threonine 668 as well as some β-amyloid fragments [19, 20]. Phosphorylation in cells is a tightly regulated process often acting as a molecular switch. Because DYRK1A is up-regulated in DS and phosphorylates many proteins such as Tau, APP and Aβ, essential neuronal processes including differentiation, APP processing, and Tau phosphorylation are negatively impacted (Fig. 2) [20].

Figure 2: Summary of the role of DYRK1A Overexpression in DS. Schematic of the neurological consequences due to the overexpression of DYRK1A. The hyperphosphorylation of NFAT, APP, Tau, and α-synuclein are due to the increased production of DYRK1A. Increased DYRK1A promotes its own overexpression by
increasing the amount of β-amyloid production via phosphorylation of APP. Figure reproduced from., [20].

DYRK1A can also function as a regulator of basic molecular metabolism, which is achieved by DYRK1A phosphorylating eukaryotic protein synthesis initiation factor 2B epsilon (eIF2Bε) and glycogen synthase [21]. The effects that DYRK1A has on eIF2Bε and glycogen synthase give rise to the theory that this gene can regulate the cellular cycle. Whether or not DYRK1A modulates the cellular cycle is debatable, but this gene is widely involved in many essential cellular processes.

1.2,3 Ets-2

Ets-2, is a gene also located on HSA21 and has been suggested to be involved in the pathogenesis of DS. Ets-2 is a transcription factor involved in many cellular processes such as differentiation, maturation, and activation of certain signaling cascades [22]. The role that ets-2 plays in DS is thought to be one of a harmful protein which, when its expression is increased, predisposes cells to undergo apoptosis via a p53-dependent pathway [23]. The increased expression of ets-2 leads to apoptosis by activating p53, which promotes the upregulation of mitochondrial pro-apoptotic factors Bax and apoptotic activating factor-1 (Apaf-1). The activation of p53 inhibits the transcription of pro-survival proteins including B-cell lymphoma-2 (Bcl-2) and Inhibitors of Apoptosis (IAPs) [24]. In DS ets-2 overexpression is associated with bax activation, intracellular Aβ accumulation, and hyperphosphorylation of tau, suggesting that ets-2 overexpression may contribute
to the neuronal loss and degradation that Individuals with down syndrome experience.

The antioxidant enzyme SOD1 is also located on the q arm of HSA21 and has been found to be overexpressed by ~50% in DS individuals. The function of SOD1 is to catalyze the dismutation of superoxide anions to hydrogen peroxide. The increased expression of this enzyme may lead to an increase in reactive oxygen species in DS [25]. Mis-folded or mutated SOD1 has been studied as the cause of the disease amyotrophic lateral sclerosis (ALS) also known as Lou Gehrig’s disease. SOD1 has also been studied in relation to the gene dosage theory in DS. SOD1 overexpression is implicated in increased lipid peroxidation and cellular oxidative damage [26]. Cellular respiration and metabolism are necessary for life, but the by-products of these processes produce ROS. These ROS are also known as free radicals and include superoxide ($O_2^-$), hydroxyl radical ($OH^-$), hydrogen peroxide ($H_2O_2$) and peroxynitrite ($NO_3^-$). Hydrogen peroxide and peroxynitrite can be converted to highly reactive molecules through the Fenton reaction which converts $H_2O_2$ to $OH^-$ and $NO_3^-$ to $OH^-$ as well [14]. These highly reactive molecules are then able to react and damage nuclear and mitochondrial DNA often leading to apoptosis. SOD1 works as a first line cellular antioxidant defense by catalyzing the dismutation of $O_2^-$ to molecular oxygen ($O_2$) and hydrogen peroxide (Fig. 3). Hydrogen peroxide is then converted to water by catalase (CAT) and glutathione peroxidase (GPX) [27].
Figure 3: Mechanism of SOD1 Breaking Down Superoxide. The expression of 1.5 times its normal level in Individuals with down syndrome causes an imbalance in the ratio of SOD1 to that of CAT and GPX resulting in an accumulation of H$_2$O$_2$. Figure Reproduced from., [28].

Oxidative stress and mtDNA damage can cause death in all cells, but neurons are more susceptible to ROS. Fetal loss of unnecessary neurons is common, but the loss of functional neurons because of ROS is detrimental to a developed brain due to the fact that neurons are post mitotic. Elevated levels of SOD1 in DS brains most likely plays a significant negative role because of the contribution to the increased production of hydrogen peroxide leading to an increase of the Fenton reaction producing greater levels of OH$^\cdot$. 

\[
\begin{align*}
(O_2^\cdot) & \xrightarrow{\text{SOD1}} (H_2O_2) \\
& \xrightarrow{\text{Fenton Reaction}} OH^\cdot \\
\text{CAT} & \quad \text{GPX} \\
\xrightarrow{\text{H}_2\text{O} + \text{O}_2} & \quad \xrightarrow{\text{H}_2\text{O} + \text{OH}^\cdot} \text{Oxidative damage} \\
\text{•DNA} & \quad \text{•mtDNA} \\
& \quad \text{•Protein} \\
& \quad \text{•lipid}
\end{align*}
\]
1.3 Amyloid Precursor Protein

Figure 4: Ribbon Structure of the Amyloid Precursor Protein. (www.pdb.com)

APP, often referred to as the Alzheimer’s gene is most frequently cited as a potential cause of the global dementia and neuronal loss seen in individuals with DS (Fig. 4). Also located on HSA21, this gene is repeatedly found upregulated in individuals with DS as well as the accumulation of its cleavage product, β-amyloid, which forms neuronal plaques and has been found as early as 8 years of age in DS patients [29]. APP has several isoforms ranging from 365 to 770 amino acid residues [30]. APP is cleaved by three proteins termed α-, β- and γ-secretases (Fig. 5). The cleavage of APP by α- and β-secretase releases large soluble proteins titled APPsα and APPsβ respectively [31]. These two soluble proteins are then cleaved
again by $\gamma$-secretase. Cleavage of APPs$\alpha$ by $\gamma$-secretase produces a 3 kDa product entitled p3, whereas the cleavage of APPs$\beta$ by the same $\gamma$-secretase releases the well known A$\beta$ fragment. Along with the production of either p3 or A$\beta$ from the cleavage of APPs$\alpha$ and APPs$\beta$, $\gamma$-secretase also produces from both precursor proteins, the APP intracellular domain (AICD). AICD and p3 are essentially innocuous when it comes to plaques and dementia; however the cleavage product of APPs$\beta$, A$\beta$, is responsible for the formation of plaques and ultimately neuronal loss. There are two isoforms derived from the cleavage of APPs$\beta$, the shorter being A$\beta$(1-40 amino acids) and the longer more neurotoxic form, A$\beta$(1-42 amino acids). There are two initial cleavage products of APP, previously listed, but the factors that promote the generation of one product over the other are unclear. One such factor appears to be the gene $\beta$-amyloid cleaving enzyme (BACE2) which is also located on HSA21. Although it is not typically overexpressed at the 1.5x level observed for APP it is still found to be somewhat up-regulated in DS. The increased activity of this enzyme promotes the formation of the APPs$\beta$ fragment, thus increasing of the release of the neurotoxic A$\beta$ fragment [32].
DS and AD are linked in many ways, but the most notable being that by the age of 40, virtually all individuals with DS exhibit the neuropathology of AD [33]. APP is a ubiquitously expressed transmembrane type I protein, found in many tissues, but is concentrated in the synapses of neurons [34]. The cellular location of APP gives rise to the theory that APP may play a part in the formation and regulation of synapses and neural plasticity [35]. Although APP itself has certain roles in the brain, the neuronal pathology of AD is more closely associated with its cleavage product, Aβ.

APP is overexpressed in Individuals with Down syndrome and this inevitably leads to an increased amount of soluble amyloid-β in the brain. The increase of Aβ
fragments promotes self-aggregation leading to the formation of plaques. Aβ fibrils are cytotoxic to neurons due to Aβ’s ability to trigger cells to generate oxygen free radicals [36]. Experimental data suggests that monomers, oligomers, and fibrils of Aβ negatively impact proliferation and differentiation of neural progenitor cells (NPCs). If Aβ suppresses cellular proliferation and differentiation, then the observed overexpression of APP in DS is a likely contributor to the neuronal loss and early onset AD that DS patients occasionally develop. Aβ also induces oxidative stress by activating microglia, increasing neuronal calcium influx (Ca²⁺), and inducing apoptosis [37]. An increase in intracellular calcium has been implicated in activating the intrinsic apoptosis pathway.

Normal brain development involves the generation of neurons and extensions of axons, neuronal pruning then follows this phase so that appropriate connections are strengthened and maintained. Widespread loss of neuronal connections underlies the neurophysiology of DS and AD in DS. When neurons are destined for degeneration they are often targeted by extrinsic signals or intrinsic factors that promote apoptosis. DR6, also known as tumor necrosis factor receptor superfamily, member 21 (TNFRSF21), can regulate neuronal death and axonal pruning [38]. If neurotrophic factors are insufficient, DR6 will trigger neuronal cell body and axon degeneration by activating a Bax and caspase-3-dependant apoptosis cascade [39]. Nikolaev 2009 reported that the extracellular fragment of APP acts as a ligand for DR6 indicating that APP itself is sufficient to trigger neuronal cell death and axonal pruning [38].
Overexpressed APP has indirect negative effects on mitochondria as a result of the increased ROS and increased intracellular calcium levels, but APP also has a direct impact on the dynamics of mitochondria. APP carries a mitochondria-targeting signal sequence in its N-terminal domain, which causes APP to insert itself into mitochondrial import channels [40]. APP has been found in the mitochondria of cortical neurons of AD transgenic mice and when overexpressed in cultured cells, APP is once again found within mitochondria [41]. When APP accumulates in the mitochondria, these organelles then become susceptible to swelling as well as leaking ROS into the cytosol an in turn causes intrinsic apoptosis [42].

APP overexpression has negative impacts on normal neuronal and cellular processes. These are achieved through the production of ROS, disruption of mitochondrial dynamics and accumulation of toxic plaques. Because APP is overexpressed in Individuals with Down syndrome it is likely that APP plays a significant role in the neuronal loss observed in the hippocampus and cortex of DS individuals.

1.4 Mitochondrial Oxidative Stress and Lipid Peroxidation

Some of the genes located on HSA21, such as ets-2, SOD1, RCAN1 and APP have been implicated in increased production of ROS leading to cellular and mitochondrial oxidative stress in DS individuals. Mitochondrial DNA (mtDNA) is 10 times more vulnerable to oxidative damage in vivo compared to that of nuclear DNA; the increased production of ROS that these proteins produce is especially
harmful when overexpressed in DS [14]. Although mitochondria are very susceptible to ROS, because of cellular respiration they are also large producers of the very same ROS. Due to their high affinity to oxidize cellular components, ROS often cause DNA double stranded breaks (DSBs). Current theories suggest that mild DNA stress will slowly induce impairment, often seen in ageing, whereas acute oxidative stress triggers the mechanisms involved in cellular death [43].

The damage to mtDNA that is induced by increased levels of ROS leads to mutations in mtDNA-encoded proteins [44]. MtDNA is more susceptible to ROS because there are no histones to protect the DNA, and because mitochondrial are the largest producers of ROS in a cell [40]. The errors in the mtDNA cause defects in the mitochondrial complexes that are involved in the electron transport chain. If one of these complexes is mis-folded or contains mutations then its ability to successfully shuttle highly reactive electrons between other complexes in the mitochondrial inner membrane become inhibited, leading to an increase of ROS.

Along with oxidizing mtDNA, ROS can also cause damage to the lipid membranes of cells. Because lipids are critical to the structure and function of cells, any disruption to their function can lead to cell death. The double bonds found in polyunsaturated fatty acids (PUFAs) which help form the membranes of cells are ready targets for free radical attack [45]. The hydrogen atoms in PUFAs are most susceptible to peroxidation by free radicals. Once this hydrogen is oxidized a chain reaction is set into motion by the creation of a new radical molecule. The newly created molecule is then able to remove another hydrogen from another PUFA [46].
When a large portion of lipid molecules are disrupted the function of ion channels, and other membrane proteins are also disrupted affecting the entire lipid membrane and homeostasis of the cell.

Cells do contain molecules that inhibit the destructive nature of ROS, the most abundant of which is glutathione (GSH), which is the tri-peptide, \(\gamma\)-glutamyl-cysteinyl-glycine. Anti-oxidant GSH, in the company of GPX reacts readily with ROS generating the glutathiyil radical (GS\(^\bullet\)) which is much less oxidizing than the original molecule [47]. This GS\(^\bullet\) radical rapidly reacts with another GSH molecule to form oxidized glutathione (GSSG). The donation of hydrogen by GSH maintains the important balance of ROS and oxidized molecules. Mitochondria are unable to synthesize GSH, therefore mitochondrial GSH originates in the cytosol and is transported into mitochondria [48]. When mitochondrial GSH is depleted, these organelles become extremely susceptible to oxidative stress. The danger of mitochondrial oxidative stress lies downstream of the mitochondria itself. It is the pro-apoptotic molecules such as cytochrome c that are contained within the mitochondria that cause widespread cellular damage.

In the erythrocytes of AD individuals, GSH levels have been found to be decreased, along with a corresponding downregulation of glutamate-cysteine ligase (GCL) and glutathione synthase (GS), the two enzymes involved in \textit{de novo} GSH synthesis [49]. Reports have also found that in individuals with down syndrome
levels of GSH are also markedly reduced [50]. With the reduction in this critical anti-oxidant that both AD and DS patients exhibit, it is only a matter of time until certain cells are overcome by normal mitochondrial respiration and production of ROS. Normal aging is thought to arise from cells that are overcome by ROS; it seems that cells from these DS and AD individuals succumb to this much faster or that these afflicted cells are less prepared for ROS increases due to the reduction of GSH.

1.5 Intrinsic Apoptosis Pathway

There are two apoptotic pathways the extrinsic and intrinsic. The extrinsic apoptosis pathway is activated when the Fas ligand binds to its extracellular receptor which activates caspase-8 eventually leading to apoptosis. The intrinsic apoptotic pathway is initiated through specific activation of pro-apoptotic molecules that are normally sequestered within the mitochondria of healthy cells. The signals that typically activate this pathway result from oxidative stress, detachment from the extracellular matrix, a defective cell cycle, hypoxia, or a loss of cell survival factors [51]. Mitochondria are key players in the intrinsic apoptosis pathway and activation of this pathway is caused by the permeabilization of the outer mitochondrial membrane (Fig. 6). When this occurs pro-apoptotic molecules such as cytochrome c are released into the cytosol where they activate cystiene-dependent, aspartate-specific proteases (*caspases*). Caspases are zymogens that upon cleavage are activated and able to cleave essential cellular proteins propagating the steps involved in apoptosis.
When permeable mitochondria release cytochrome c the apoptosome is formed, a large oligomer complex composed of cytochrome c, Apaf-1, and caspase-9. Cytochrome c binds to the C-terminal region of Apaf-1, allowing Apaf-1 to oligomerize and become a scaffold for caspase-9 [52]. Caspase-3 is activated by the apoptosome through cleavage by caspase-9 and is known as the executioner caspase. Activated caspase-3 then moves on to cleave key proteins in the cell causes the cellular and biochemical events of apoptosis [51].

Mitochondrial swelling and enhanced outer membrane permeability promotes apoptosis through an alternative route that also activates the intrinsic apoptotic pathway, titled the mitochondrial permeability transition pore (mtPTP). This pore is thought to be composed of the voltage-dependent anion channel (VDAC), members of the Bax-Bcl-2 protein family, cyclophilin D, and the adenine nucleotide translocator (ANT) [53]. In earlier sections, it was noted that oxidative stress and increases in intracellular Ca²⁺ have been implicated in neurological diseases. Normal respiring mitochondria take up calcium ions; however, if excessive calcium ions are incorporated into the mitochondria then mitochondrial matrix swelling occurs and the mtPTP is susceptible to being activated. Upon activation of the mtPTP, VDAC turns into a non-selective pore where it allows ions and small molecules (∼1500 Da) to enter the matrix. Due to the large surface area in the matrix of mitochondria, swelling of this compartment causes rupture of the organelle allowing cytochrome c to escape mitochondria and activate the intrinsic apoptotic cascade [54].
Bax is a pro-apoptotic cytosolic protein that is a member of the B-cell lymphoma protein family. When activated, bax localizes to the outer mitochondrial membrane and oligomerizes to form pores. Bax is normally a monomeric protein and is unable to form channels in mitochondria but when activated, usually by truncated BID (tBID) a Bcl-2 Homology-3-only (BH3-only) pro-apoptotic protein, is able to oligomerize and form channels. Oligomeric bax forms a pore that is between 2.7-5.4 nm wide which is sufficient to allow the passage of ~3 nm cytochrome c though the pore and into the cytosol [55, 56]. These bax channels are also voltage independent and are fairly cation selective, thus small molecules including calcium are able to enter the mitochondrial inner membrane space and cause swelling. The channels formed by bax can be inhibited by anti-apoptotic members of the Bcl-2 family. Bcl-2 itself is able to prevent bax activated apoptosis as well as mitochondrial oxidative stress.

Bak is another pro-apoptotic member of the Bcl-2 family. Unlike bax, bak resides on the outer membrane of mitochondria. Bak is an effector molecule for mitochondrial outer membrane permeabilization (MOMP) [57]. Bak is activated when tBID migrates to the mitochondrial membrane and recruits bax to the same location. When bax is activated it once again inserts itself into the membrane forming homo (bax/bax) or hetero (bax/bak) dimers forming the MOMP complex [58]. The oligomerization of Bax and bak forms the mitochondrial apoptosis-induced channel (MAC) which allows cytochrome c to be released from mitochondria (Fig. 6) [59].
Figure 6: Intrinsic Apoptosis Cascade. tBid activates bax and bak increasing mitochondrial outer membrane permeabilization. ROS promote the activation of these two molecules, but bax/bak effects can be attenuated by Bcl-2. Released cytochrome c activates APAF-1 → caspase-9 → caspase-3 → apoptosis.

1.6 Hypothesis and Rationale

Previous studies have shown that APP causes an increase in intracellular calcium as well as disruption of normal mitochondrial dynamics. We hypothesized that due to the overexpression of APP, cells will undergo apoptosis through an
intrinsic apoptotic cascade induced by increased intracellular levels of calcium that initiate the process of triggering the mtPTP.

APP is overexpressed in DS individuals, therefore we assessed whether APP’s effects on mitochondrial oxidative stress and activation of pro-apoptotic factors could be prevented by antioxidants or anti-apoptotic molecules. If this is the case, then our findings would suggest that individuals with DS and AD might benefit from such strategies aimed at mitigating the neurotoxic effects of APP.

1.7 Summary of Major Findings

Using a transient transfection strategy of APP in combination with fluorescence imaging and western blot analysis we detected a significant elevation in apoptosis, and mitochondrial oxidative stress due to the overexpression of APP in CHO cells. Demonstration of the release of cytochrome c from mitochondria solidified our hypothesis that APP was causing activation of the intrinsic apoptotic cascade.

Along with the detection of apoptosis through the use of western blotting and fluorescence imaging we were able to demonstrate a marked increase in ROS and mitochondrial oxidative stress through the use of glutathione assays and a mitochondrial permeability transition pore assay.

Finally, we showed that antioxidant (exogenous glutathione) and anti-apoptotic (caspase inhibitors or Bcl-2 overexpression) were strategies that provided significant protection from APP-induced toxicity.
Collectively, these novel findings indicate that APP overexpression likely plays a deleterious role in DS via the induction of mitochondrial oxidative stress that is sufficient to cause activation of the intrinsic apoptosis cascade. Future studies aim to replicate the findings in this study in a more relevant neuronal system.
CHAPTER TWO: MATERIALS AND METHODS

2.1 Reagents

4,6-diamidino-2-phenylindole (DAPI), and Hoechst Dye number 33258 were purchased from Sigma (St. Louis, MO). Glutathione monoethyl ester (GSH) was purchased from Calbiochem (San Diego, CA). The pan-caspase family inhibitor Boc-D (OMe)-FMK was from Alexis Biochemicals (Plymouth Meeting, PA). Caspase 3 antibody was purchased from Promega (Madison, WI). Horseradish peroxidase-conjugated secondary antibodies and reagents for chemiluminescence detection were purchased from Amersham Biosciences (Piscataway, NJ). DsRed-2 was obtained from Invitrogen (Carlsbad, CA). Image-iT live mitochondrial transition pore assay kit was purchased from Invitrogen (Carlsbad, CA). Cytochrome C antibody was purchased from Biolegen (San Diego, CA). APP antibody was purchased from Abcam (Cambridge, MA). COX-IV antibody was purchased from Cell Signaling (Boston, MA). Bax-6A7 antibody was purchased from Alexis Biochemicals (Plymouth Meeting, PA). Cyclosporin A was purchased from Alexis Biochemicals (Plymouth Meeting, PA). Maxi-prep plasmid kits were purchased from (Valencia, CA). Kanamycin and Ampicillin were purchased from Sigma Aldrich (St. Louis, MO). F-12 Ham CHO cells media was purchased from Sigma Aldrich (St. Louis, MO). Lipofectamine-2000 was purchased from Invitrogen (Carlsbad, CA). Opti-Mem was purchased from Invitrogen (Carlsbad, CA). JM109 competent E. Coli was purchased from Promega (Madison, WI). Staurosporine was purchased from Sigma Aldrich (St. Louis, MO). Mitochondrial/Cytosolic fractionation kit was purchased from Bio
Vision (Mountain View, CA). Glutathione assay kit was purchased from Oxford Biomedical (Oxford, MI).

2.2 CHO Cell Culture

K1-CHO cells were a generous gift from Dr. David Patterson. Cells were plated on 35-mm diameter plastic dishes in F12-Ham’s media containing 10% fetal bovine serum, 2mM 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 CHO Cell Transfection

Plasmids encoding DsRed-2 or DsRed-APPwt proteins were transfected at a concentration of 5µg of DNA. Co-transfection with Bcl-2 plasmid utilized 10µg of DNA. 50µl of Opti-mem minus the pre-calculated volume of plasmid was incubated at room temperature for 10 minutes. Correspondingly, 45µl of Opti-mem and 5µl of lipofectamine-2000 reagent were incubated at room temperature for 10 minutes. The mixture of lipofectamine-2000 and Opti-mem were then mixed together and incubated for 20 minutes at room temperature. During the 20 min incubation period the 6-well dishes of CHO were and washed twice in 37°C Opti-mem. Final volume of Opti-mem for the transfection was 1ml. The 100µl of DNA-Lipofectamine-2000 and Opti-mem were then added to each well of the 6-well plate.
After which the cells were incubated for 6 h at 37ºC, 10%CO₂, and finally the Opti-
mem was replaced with 1ml of F-12 HAM media as described above.

2.4 Bacterial Transformation of DsRed-2

DsRed-2 was obtained from Clontech at a concentration of 500 ng/µl, the plasmid was diluted to a final working concentration of 50ng/µl. 100µl of JM109 competent Escherichia coli cells were placed in a pre-chilled falcon tube on ice. 1µl of .7M β-Mercaptoethanol was added to the cells and swirled every 2 minutes for 10 minutes. Next, 1µl of the diluted DsRed-2 plasmid was added to the E. coli cells and incubated on ice for 30 minutes. After the 30 min incubation cells were heat shocked for 45 s and incubated on ice for 2 m. Next, 900µl of LB broth with Ampicillin at a concentration of 100µg/ml was added to the tube. The tube containing the cells and broth was shaken at 200 RPMs at 37º C for 1 h. Next 50µl of cells were plated on an LB agar plate containing 100µg/ml Ampicillin and spread using a sterile glass pipette. The agar plate was then incubated at 37ºC degrees overnight.

2.5 Bacterial Transformation of DsRed-APPwt

DsRed-APPwt was the kind gift of Dr. Xiongwei Zhu at a concentration of 50 ng/µl. DsRed-APPwt was transformed as stated above.

2.6 Bacterial Transformation of Bcl-2

Bcl-2 plasmid was transformed as stated above.
2.7 *DsRed-2, DsRed-APPwt and Bcl-2 Plasmid Preparation*

After plasmid transformation, individual colonies were selected from the agar plates using a 10µl pipette and incubated in 5ml of LB broth with Ampicillin 100µg/ml (DsRed-2, Bcl-2) or Kanamycin (DsRed-APPwt) 40µg/ml for 6-8 h at 37°C shaking at 200RPMs. After the 6 h were complete, starter cultures were diluted 1:250 and incubated over night. Plasmids were prepared using a Qiagen endo-free Maxi-prep kit. Cultures were pelleted and collected in a 250ml bottle and spun at 4°C at 10,000 x g for 30 minutes. The supernatant was removed and the cells were re-suspended in pre-chilled 10ml buffer P1 (50 mM Tris HCl pH 8.0, 10mM EDTA, 10 µg/ml RNASE A). 10ml buffer P2 (200mM NaOH, 1% SDS, 1 M NaOH, 10% SDS, H2O) was added to mixture and incubate at room temperature for 5 minutes. Next, pre-chilled buffer P3 (3.0 M KOAc pH 5.5) was added to the mixture and poured into the barrel of the cartridge. The mixture incubated at room temperature for 10 minutes at which point a plunger was inserted into the barrel and lowered with moderate force transferring approximately 25ml of supernatant to a 50ml polycarbonate tube. 2.5ml of buffer ER (endotoxin removal) was added and inverted 4-6 times and incubated on ice for 30 minutes. As the mixture incubated on ice, a separate Qiagen-500 filter tip was equilibrated with 10ml of buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% isoproponol and .15% Triton X-100). Next the mixture was poured into the Qiagen-500 filter tip and filtered by gravity. The filter was washed two times with 30ml of Qiagen buffer QC (1.0 M NaCl, 50 mM MOPS pH 7.0, 15% isopropanol) by gravity flow. Plasmid DNA was eluted into 50ml
polycarbonate tube by filtering adding 15ml of buffer QN (1.6 M NaCl, 50 nM MOPS pH 7.0, 15% isopropanol) to the filter tip. DNA was precipitated with 10.5ml of 100% isopropanol to eluted DNA and centrifuged at 4ºC for 30 minutes at 15,000 x g. The approximately 30ml supernatant was decanted and the pellet was resuspended in 5ml endotoxin-free 70% ethanol, and centrifuged for 10 minutes at 15,000 x g at 4ºC. The 5ml supernatant was decanted and the pellet was resuspended in 200µl of buffer TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA) and allowed to air dry. Upon retrieval of plasmid/buffer mixture 1.5 µl of sample was analyzed using Thermo Scientific NanoDrop 2000 to determine DNA concentrations.

2.8 Bcl-2 Bacterial Transformation

Bcl-2 bacterial transformation was prepared using the exact protocol of the DsRed-2 plasmid. See previous DsRed-2 protocol.

2.9 Treatments

Immediately following transfection cells were supplemented with specific treatments and incubated for ~24 h.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boc- pan caspase inhibitor</td>
<td>50µM</td>
</tr>
<tr>
<td>Glutathione- endogenous antioxidant</td>
<td>2mM</td>
</tr>
<tr>
<td>Staurosporine- Bax activator</td>
<td>500nM</td>
</tr>
</tbody>
</table>
Cyclosporine A - Immunosuppressant drug  

<table>
<thead>
<tr>
<th>Antibody or Stain</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>4′,6-diamidino-2-phenylindole (DAPI)-Chomatin stain</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table I: Treatments that were used for CHO Cell Experiments.

2.10 Immunocytochemistry/Fluorescent Imaging

24 h post-transfection and treatments, cells were washed once in 1x PBS and then fixed with 1ml of 4% Paraformaldehyde for 45 min at room temperature. Cells were then permeabilized and blocked with 5% BSA in 0.2% Triton X-100/1xPBS at room temperature for 1 h. Primary antibodies were diluted as in table II in 2% BSA in 0.2% Triton X-100/1xPBS, and 1ml was added to each well and incubated overnight at 4ºC. The following day cells were washed 5x with 1X PBS, and secondary antibody at a dilution of 1:250 in 2% BSA in 0.2% Triton X-100/1X-PBS was prepared and 1ml was added per well for 1 h incubation at room temperature. Next, cells were washed 5x in 1X PBS, and placed in 1ml of an anti-quench solution prepared from 1X PBS and 1-5 mg of p-phenylenediamine. For DAPI staining only, cells were incubated for 1 h at room temperature in 2% blocking solution as previously stated. Fluorescent images were captured using a 50x objective on a Zeiss Axiovert microscope.
Table II: Antibodies And Stains Utilized for Experiments.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hoechst- Chromatin stain</td>
<td>1:1000</td>
</tr>
<tr>
<td>Anti-Active Caspase-3 pAb</td>
<td>1:1000</td>
</tr>
<tr>
<td>6A7-Anti-Active Bax pAb</td>
<td>1:200</td>
</tr>
<tr>
<td>FITC-Anti Rabbit</td>
<td>1:250</td>
</tr>
<tr>
<td>FITC-Anti Mouse</td>
<td>1:250</td>
</tr>
</tbody>
</table>

2.11 Subcellular Fractionation

A Mitochondrial/Cytosolic fractionation kit was obtained from BioVision, Inc. Buffers for each fractionation were prepared as follows: 1ml of cytosolic buffer was diluted in 4ml of ddH₂O, 10µl protease inhibitors, and 5µl of dithiothreitol (DTT) were added. 2µl of protease inhibitors and 1µl of DTT were added to 1ml of the 1X mitochondrial buffer. Cells were washed 1x with ice-cold 1XPBS. The 1XPBS was aspirated and 200µl of cytosolic extraction buffer added per well and incubated on ice for 20 min. After which cells were scraped, harvested, and transferred into a microcentrifuge tube. Lysates were then Dounce homogenized ~40 times. Heavy membrane lipids were fractioned by spinning at 4°C for 10 minutes at 720 relative centrifugal force (RCF). Supernatant was transferred to a clean microcentrifuge tube and mitochondria were fractionated by centrifugation at 10,000 RCF for 30 min. Next, the supernatant was transferred to new tube. The mitochondrial pellet was resuspended in 100-150µl of mitochondrial extraction buffer.
2.12 Cell Lysis and Immunoblotting

Post transfection and treatment cells were washed 1 time with 1X ice cold PBS; pH 7.4 and were incubated for 20 minutes on ice with 200µl of cytosolic extraction buffer. Samples were scraped from the wells and combined into a microcentrifuge tube. Protein concentration was determined using a protein assay kit; (BCA, Pierce Chemical Co., Rockford, IL) and 60µg of protein were electrophoresed through polyacrylamide gels, and resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences, Piscataway, NJ). Immunoblotting was performed by blocking PVDF membranes in 1X PBS; pH 7.4 with 0.1% Tween 20 (PBS-T) containing 1% BSA and 0.001% sodium azide for 1 h at room temperature. The primary antibody was diluted in blocking solution according to manufactures recommendations and incubated with membrane for 1 h. Membranes were then washed 5x over 30 min with PBS-T. Next, membranes were incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies diluted in PBS-T, following, membranes were washed 5x over 30 min with PBS-T. This was followed by detection of immunoreactive proteins with enhanced chemiluminescence.

2.13 Glutathione Assay (Oxford Biomedical Research, GT-35)

An Oxford Biomedical Research glutathione kit was used, but solutions were scaled to 96-well plate reader dish. After mitochondrial/cytosolic fractionation was performed as described above. All kit buffers were made per manufacturer’s
instructions. 30µl of pre-made standards were added to the 96-well plate. 30µl of pre-made reductase with DTNB and 30µl reductase for 10 min was added to each well including sample wells. During which samples were added to the 96-well plate at a 1:5 dilution. Next, 30µl of NADPH was added to standards, and 30µl DTNB was added to samples. Absorbance was read at 412nm in a Bioteck Spectrophotometer.

2.14 Image-IT Live PTP Assay (Invitrogen Kit, I35103)

24 h post transfection, CHO cells were washed 1x with Hank’s balanced buffered salt solution (pH 7.4), and cells were placed in 997µl of Hank’s buffer. 1.0mM calcein AM, 1.0mM Hoechst 33342, and 1.0M Cobalt (II) chloride hexahydrate were added at 1µl each per well of cells. Cells were incubated at 37ºC incubator at 10% CO₂ and incubated for 15 minutes. Upon completion of the 15-minute incubation cells are washed 1x with Hank’s balanced buffered salt solution (pH 7.4) and live imaged under fluorescence microscopy.

2.15 Statistical Analysis and Quantification of Data

Recorded values of apoptosis, caspase-3 activation, DNA fragmentation and, mitochondria permeability transition pore opening were quantified from 30 images per treatment. Graphical data represent the mean ±SEM for the respective experiments. Only transfected cells were counted in each field disregarding untransfected cells. Values are shown as a percentage of total transfected cells. Most experiments are a representative of an n=3 value. Statistical analysis of these
experiments was performed using GraphPad Prism, each respective experiment was
analyzed for significance with a 1-way analysis of variance (ANOVA) and a post hoc-
Tukey's test. Accepted significance had a p value <0.05.

Glutathione assays raw data was analyzed by exporting the kinetic data to
Microsoft Excel. The kinetic data was then averaged to fit a standard curve, only R²
values greater than .950 were accepted. Slope of curves were used as kinetic rate of
reduced GSH concentration and were normalized to previously determined sample
protein concentrations. These values are all represented as a percentage of control
(DsRed-2).
CHAPTER THREE: RESULTS

3.1 Overexpression of Amyloid Precursor Protein Induces Apoptosis in CHO Cells

To explore the effects of APP on apoptosis in CHO cells we used 60-80% confluent cells and transfected them with an APP plasmid for 6 h. The cells were then incubated for an additional 24 h before imaging. Transfection efficiency was approximately 30-50% as assessed by DsRed fluorescence. We also transfected cells with an empty DsRed-2 vector as a control to ensure that the plasmid and or transfection procedure were not causing the cells to undergo apoptosis. Cells were fixed and stained with DAPI to image nuclear morphology. Interestingly, CHO cells that overexpressed APP showed distinct fragmented or condensed nuclei indicative of apoptosis whereas, CHO cells that only expressed the empty vector had normal nuclear morphology (Fig.7 & 8).
Figure 7: Overexpression of APP induces nuclear condensation and fragmentation in CHO cells. Panel A: DsRed-2 (red) transfected cells with nuclei DAPI (blue) stained. The nuclei are round and intact indicating a healthy cell. Panel B: APP (red) transfected cell with nuclei DAPI (blue) stained. The nuclei are fragmented lacking order, indicating apoptosis. Panel C: APP (red) transfected cell with nuclei DAPI (blue) stained. The nucleus is condensed indicating apoptosis.

3.2 Overexpression of APP Activates Caspase-3

Because apoptosis is mediated by caspases we next wanted to determine whether or not APP overexpression causes apoptosis though a caspase-dependent mechanism in CHO cells. Cells were once again transfected for 6 h and incubated for an additional 24 h. Following incubation, cells were fixed and stained with DAPI
to show nuclear morphology and then co-stained with a rabbit polyclonal antibody against the active form of caspase-3. Using an anti-rabbit antibody conjugated to a FITC tag, the cells were imaged using a Zeiss Axiovert 200 EPI Fluorescence inverted microscope. Compared to DsRed-2, APP overexpressing CHO cells revealed a marked increase in caspase-3 activation, indicating that APP causes activation of an apoptotic pathway (Figure 9).

![Figure 9: APP overexpression induces caspase-3 activation. Left Panel: transfected APP (red) cells, nuclei DAPI (blue) stained and active caspase-3 with a FITC (green). Right Panel: DsRed-2 (red) transfected CHO cells, stained as in previous panel.](image)

3.3 Boc, Glutathione & Bcl-2 Prevent APP Induced Apoptosis

Upon finding that CHO cell death due to overexpression of APP was through a caspase-3 mechanism, we next analyzed the nuclear morphology and chromatin
fragmentation of cells that were overexpressing APP either alone or in combination with antioxidant or anti-apoptotic agents. APP overexpressing cells were co treated with a pan caspase inhibitor (Boc), the anti oxidant glutathione, or the pro-survival protein Bcl-2. To supplement CHO cells with Bcl-2 we co-expressed a Bcl-2 plasmid, and an APP plasmid at twice the transfection concentration of the APP plasmid. Due to the fact that the Bcl-2 plasmid produces no fluorescent protein we doubled the transfection concentration of Bcl-2 over that of APP to ensure that the majority of cells expressing APP were also co-expressing Bcl-2. After 24 h post transfection, cells were fixed and stained with DAPI and imaged for nuclear morphology. APP once again caused a large proportion of the cells to exhibit nuclear fragmentation and/or condensation. Interestingly, CHO cells that were supplemented with either Boc, GSH or Bcl-2 showed very low levels of apoptosis as assessed by nuclear fragmentation (Fig. 10). The findings that Boc and Bcl-2 were each able to protect against APP-induced apoptosis led us to believe that APP caused cell death through an intrinsic apoptosis pathway. Earlier we observed that APP activated Caspase-3, the executioner caspase, and that the only cells consistently undergoing apoptosis were ones expressing APP. The protection that was observed when cells were treated with GSH was mechanistically distinct from that of Boc and Bcl-2. Notably, the intrinsic anti-oxidant properties of GSH protect against oxidative stress and perhaps in APP treated cells, specifically mitochondrial oxidative stress. In conclusion Bcl-2, GSH and Boc all maintained healthy nuclei and prevented nuclear condensation in response to APP overexpression (Fig. 10).
Figure 10: Bcl-2, GSH, and Boc protect from APP induced apoptosis. Panel A: transfected APP (red) cells, nuclei DAPI (blue) stained. Panel B: APP (red) transfected CHO cells with Bcl-2 plasmid (no-color), nuclei DAPI (blue) stained. Panel C: APP (red) transflect cells treated with Boc, nuclei DAPI (blue) stained. Panel D: APP (red) transfected cells treated with GSH, nuclei DAPI (blue) stained. Arrow indicated apoptotic cells determined by nuclear morphology.
3.4 Quantification of APP Induced Apoptosis

Transfected CHO cells were identified by Ds-Red fluorescence and the averages of apoptotic cells were determined from each treatment population. When cells were transfected with DsRed-2 the population remained relatively healthy with only ~7% apoptosis. CHO cells overexpressing APP were significantly induced to undergo apoptosis at a rate of roughly 55%. The death induced by APP overexpression was reduced significantly when cells were co-treated with Boc, GSH or Bcl-2 (Fig. 11). Apoptosis was quantified initially as transfected cells whose nuclei showed fragmentation and condensation, common hallmarks of apoptosis.
Figure 11: Quantification of APP induced apoptosis and protective effects of Boc, GSH, and Bcl-2. Thirty images per treatment were captured, corresponding transfected apoptotic cells were quantified. The total apoptotic cells per treatment were divided by the total number of transfected cells counted and converted into a percent. APP significantly increased apoptosis compared to control DsRed-2 where as Boc, GSH and Bcl-2 were able to reduce apoptosis approximately to control levels. *p<0.05 APP vs. APP+Bcl-2, **p<0.01 APP vs. APP+Boc, ***p<0.001 APP vs. APP+GSH. p=0.0006, n=3

3.5 APP Overexpression Induces Nuclear Fragmentation

As a sub-study of APPs effects on nuclear morphology, we specifically examined nuclear fragmentation in CHO cells. We were surprised to find that values for DNA fragmentation were very similar to that for total apoptosis of the cells. DsRed-2
cells had very low levels of DNA fragmentation averaging ~6.5%. APP overexpressing cells showed significantly increased nuclear fragmentation compared to that of control DsRed-2 cells up to ~56%. Similar to total apoptosis, Boc, GSH and Bcl-2 each significantly reduced nuclear fragmentation due to APP overexpression (Fig.12). These findings suggest a marked activation of caspase activated DNases (CADs) in cells overexpressing APP.

**Figure 12: Quantification of APP Induced DNA Fragmentation.** Thirty images per treatment were captured, corresponding transfected cells with fragmented DNA were counted. The total fragmented cells per treatment were divided by the total number of transfected cells quantified and converted into a percent. APP significantly increased apoptosis compared to control (DsRed-2) where as Boc, GSH
and Bcl-2 were able to reduce caspase-3 activation to control levels. ***p<0.001
DsRed-2, APP+Boc, APP+GSH, APP+Bcl-2 vs. APP. p<0.0001, n=3.

3.6 APP Induced Caspase-3 Activation is Attenuated by Boc, Glutathione & Bcl-2

Apoptosis and caspase-3 activation were clearly induced by the
overexpression of APP in CHO cells. Because Boc, GSH and Bcl-2 all showed
protective effects against APP-induced apoptosis, the next logical step was to
determine whether or not these same compounds were able to prevent caspase-3
activation. This experiment was performed similarly to previous experiments. CHO
cells were transfected for 6 h with either DsRed-2 or DsRed-APP. Upon completion
of the transfection, GSH or Boc was added to the cells; alternatively Bcl-2 plasmid
was once again co-transfected with the original APP plasmid. Cells were incubated
for 24 h and then fixed and stained with DAPI and an anti-active caspase-3
described previously. Boc, GSH and Bcl-2 were each able to considerably reduce the
appearance of active caspase-3 (Fig. 13).
Figure 13: Immunocytochemistry of Boc, GSH & Bcl-2, Caspase-3 Activation. Panel A: transfected APP (red) cells, nuclei DAPI (blue) stained and active caspase-3 with a FITC (green). Panel B: APP (red) transfected CHO cells with Bcl-2 plasmid (not shown or stained), nuclei DAPI (blue) stained and active caspase-3 with a FITC (green). Panel C: APP (red) transfected cells treated with Boc, nuclei DAPI (blue) stained, and active caspase-3 with a FITC (green). Panel D: APP (red) transfected cells treated with GSH, nuclei DAPI (blue) stained, and active caspase-3 with a FITC (green).

3.7 Quantification of Caspase-3 Activation

When caspase-3 activation was analyzed quantitatively, the results were quite similar to that of apoptosis quantification. Once again all transfected cells were counted and examined for overlap of DsRed fluorescence with green (FITC) fluorescence indicating active caspase-3. DsRed-2 transfected CHO cells revealed low levels of caspase-3 activation, (~7%).Cells that were overexpressing APP had
significant levels of activated caspase-3 at a rate of approximately 56%. The activation of caspase-3 induced by APP overexpression was reduced significantly when cells were treated with either Boc, GSH, or Bcl-2 (Fig. 14).

Figure 14: Quantification of APP Stimulated Caspase-3 Activation. APP significantly increases caspase-3 activation compared to control where as Boc, GSH and Bcl-2 were able to reduce caspase-3 activation to approximately control levels. **p<0.01 APP vs. APP+GSH, ***p<0.001 APP vs DsRed-2, APP+Boc & Bcl-2. p<0.0001, n=3.
3.8 Western Blot Analysis of APP Overexpression

To ensure that APP overexpression in transfected CHO cells was not affected by Boc, GSH or Bcl-2 co-transfection, we performed western blots on whole cell lysates at 24 h post-transfection. We observed no detectable APP protein in DsRed-2 transfected cells. In contrast, cells that were transfected with APP either alone or in combination with Boc, GSH, or Bcl-2 all showed detectable and approximately equal levels of human APP protein (Fig. 15). Fascinatingly, the Bcl-2 transfected cells did not display the APP doublet that was observed in either APP or those treated with Boc or GSH control cells. It is postulated that Bcl-2 may be interacting with APP in a way that is preventing certain processing events; nonetheless, the details of this possible interaction will be studied in more detail at a later date.

![Western Blot Analysis of Human APP Overexpression](image)

*Figure 15: Western Blot analysis of Human APP Overexpression.* Human APP was detected from 95 kD to 110 kD. The upper bands are processed APP while the lower kD bands are the unprocessed APP proteins. Tubulin is shown as a loading control and detected at ~50 kD.

3.9 APP Overexpression Causes Depletion of Mitochondrial and Cytosolic Glutathione

When APP overexpressing CHO cells were supplemented with GSH immediately post-transfection they displayed a marked decrease in apoptosis and
caspase-3 activation. We theorized that oxidative stress may be involved in the cellular death that these CHO cells were experiencing in response to APP. Because GSH is the most abundant endogenous anti-oxidant in cell, measurement of this peptide is an indirect indicator of cellular oxidative stress. CHO cell transfections were performed exactly as previous described. After 24 h incubation, cells were harvested, lysed and centrifuged to separate mitochondrial fractions from that of cytosolic cellular components. The mitochondrial portion of the lysates were re-suspended in an appropriate buffer and, both the cytosolic and mitochondrial fractions were analyzed for GSH in a 96-well kinetic assay and read using a plate spectrophotometer at 412nm. The readings were then normalized to protein levels and analyzed against a standard curve of GSH.

Using DsRed-2 as our control, all other values were taken as a percentage of this control level. The experiments revealed that APP significantly depleted both the cytosolic and mitochondrial pools of GSH (Fig. 16). Cells treated with Boc were not protected from the mitochondrial loss of GSH, whereas Bcl-2 and GSH supplementation showed considerable protection of the mitochondrial GSH pool (Fig. 16A).

Glutathione levels in the cytosolic fractions were dramatically reduced when cells were overexpressing APP alone. This loss of GSH in the cytosol was blunted when cells were treated with either Boc, Bcl-2 or GSH (Fig 16B).

Boc most likely was unable to prevent GSH loss in the mitochondria because Boc prevents caspase activation which occurs down stream of mitochondrial
oxidative stress. Activated caspases cleave $\gamma$-glutamylcysteine ligase (GCL), the rate limiting enzyme in the *de novo* synthesis of GSH [60]. Boc protected the cytosolic portion of GSH because in theory the caspases in the cytosol were prevented from being activated by boc, thus inhibiting their negative impact on GCL activity and cytosolic GSH levels. Bcl-2 overexpression has been shown to elevate GSH concentrations and prevent GSH depletion during stress [60-62].
Figure 16: Quantification of Glutathione in the Mitochondria and Cytosol. DsRed-2 was used as a control. All other treatment values were compared to control values. APP and APP/Boc treated cells were unable to protect against the depletion of GSH in the mitochondria. APP/GSH and APP/Bcl-2 treated cells attenuated mitochondrial glutathione depletion. APP treated cells depleted GSH in the cytosolic portion whereas APP/Boc, APP/GSH and APP/Bcl-2 all protected against APP induced glutathione depletion in the cytosol. n=2.
3.10 Overexpression of APP Activates the Mitochondrial Permeability Transition Pore

APP caused substantial oxidative stress in CHO cells as evidenced by their reduced GSH levels, indicating that these cells had increased levels of ROS. When levels of ROS are increased, especially in the mitochondria, the mtPTP is susceptible to being activated. To determine whether or not APP induced the activation of the mtPTP we transfected CHO cells similarly to previous experiment and then used a cobalt/calcein assay to measure mtPTP opening. After 24 h incubation live cells were washed once with Hank's balanced salt solution (pH 7.4) and then Hoechst was added for nuclear staining along with cobalt chloride (CoCl₂) and calcein AM. Cobalt chloride is a molecule that localizes to the matrix of mitochondria, whereas calcein AM localizes to the cytosol. Cells were incubated in this media for 15 minutes and then washed and prepared for live cell fluorescence imaging.

If the mtPTP is activated then CoCl₂ is released from the matrix of mitochondria into the cytosol allowing it to react with and quench the fluorescence signal of calcein. This simple experiment was used as an indicator of mtPTP activation. Cells that were transfected with DsRed-2 maintained high levels of calcein staining, whereas cells transfected with APP displayed diminished levels of calcein fluorescence (Fig. 17). The results of the APP expressing cells losing their calcein staining indicates the activation of the mtPTP. When APP overexpressing cells were supplemented with Boc, once again the mtPTP was activated. In contrast,
glutathione supplementation was able to prevent the activation of mtPTP in this experiment.

Boc works down-stream of the mtPTP by inhibiting caspase activation. On the other hand glutathione is an antioxidant which was able to protect against the activation of the mtPTP by reducing the levels of ROS within the mitochondria. APP most likely caused activation of the mtPTP due to the depletion of mitochondrial GSH and the consequent increase in mitochondrial oxidative stress, which sensitizes the mtPTP to elevations in intracellular Ca\(^{2+}\) which are also caused by the overexpression of APP.
Figure 17: Immunocytochemistry of Mitochondrial Permeability Transition Pore Activation. Red fluorescence indicates mito-tracker red staining. Panel A: DsRed-2 transfected cells maintained high levels of calcein AM staining indicating the mtPTP was not activated. Panel B: APP transfected cells lost most calcein AM staining indicating activation of mtPTP. Panel C: Boc was unable to protect against mtPTP activation in APP transfected cells. Panel D: APP cells treated with GSH maintained high levels of calcein AM, displaying a protection against mtPTP activation.

3.11 Quantification of Mitochondrial PTP Activation

When the data from the mtPTP activation experiment was analyzed, DsRed-2 mtPTP activation was relatively low around 30%. When DsRed-2 transfected cells were treated with ionomycin, a positive control (images not shown), mtPTP activation increased to ~70%. Ionomycin is a calcium ionophore and when administered to healthy cells it floods them with calcium thus causing mitochondria
to swell activating the mtPTP. Ionomycin-mtPTP activation was almost indistinguishable from that observed with APP alone or APP supplemented with Boc (Fig 18.). However GSH and Bcl-2 reduced mtPTP activation in the presence of APP down to the level observed in mock transfected cells.

**Figure 18: Quantification of Mitochondrial Permeability Transition Pore Activation.** Ionomycin, APP and APP/Boc treated cells all significantly activated the mtPTP. APP cells treated with GSH or expressing Bcl-2 had similar to control levels of mtPTP activation. **p<0.01 DsRed-2, APP+GSH & APP+Bcl-2 vs. DsRed-2 + Ionomycin, APP & APP+Boc. p<0.0003, n=3."
3.12 APP Overexpression Causes Mitochondrial Release of Cytochrome C

The release of cytochrome c is one of the initiating steps involved in the intrinsic apoptosis pathway. To ensure that the mechanism of death we were detecting involved cytochrome c release we performed a western blot analysis on transfected cells. In this experiment we separated the cells into mitochondrial and cytosolic fractions. Because cytochrome c is normally found within mitochondria we could be sure that cytochrome c was being released from mitochondria if it was present in the cytosolic fractions of our cells. When we examined cells transfected with DsRed-2 there was no detectable cytochrome c in the cytosol; these results were anticipated due to the low levels of apoptosis and caspase activation that we observed in previous studies. When we transfected CHO cells with APP there were detectable levels of cytochrome c in the cytosolic and the mitochondrial portions of our lysates (Fig. 19). This indicates that APP overexpression causes the release of cytochrome c from the mitochondria into the cytosol. Although the levels of detected cytochrome c in the cytosol were not as pronounced as that in the mitochondrial fractionation, one must take into account that transfection efficiency is not 100%, so the cytochrome c observed in the cytosolic portion is most likely a true representation of the relatively small population of APP transfected CHO cells that are actually releasing cytochrome c. Interestingly, when APP transfected cells were supplemented with GSH the release of cytochrome c into the cytosol was reduced, similar to the results previously observed in that GSH reduces oxidative stress and apoptosis due to APP overexpression.
3.13 APP Causes Apoptosis Through a Bax-Independent Mechanism

Given the fact the bax is inherently involved in the intrinsic apoptosis pathway we wanted to determine whether or not bax was implicated in the death pathway that APP overexpressing CHO cells were experiencing. To study this question we transfected CHO cells with APP as previously described. After the 24 h incubation, cells were washed, fixed and stained with DAPI and a 6A7-monoclonal mouse antibody against the active conformation of bax. Concurrently, as a control untransfected CHO cells were treated with the known bax activator, staurosporine. Cells were prepared for fluorescence imaging and to our surprise, the APP overexpressing cells displayed very little-to-no active bax staining compared to that of staurosporine treated cells which fluoresced brightly indicating widespread active bax staining (Fig 20.). Although the APP transfected cells did not indicate bax
activation the same cells were still experiencing apoptosis which was visualized by the nuclear fragmentation and condensation. These results indicate that APP overexpression is sufficient to cause apoptosis, but it is achieved through a bax independent mechanism.

Figure 20: Immunocytochemistry of Bax Activation. Panel A: CHO cells transfected with APP (red) and counterstained with DAPI (blue) and 6A7-anti-bax with FITC (green). Panel B: CHO cells treated with 500 nM Staurosporine, stained with DAPI (blue) and 6A7-anti-bax with FITC (green).

3.14 Cyclosporin A May Prevent APP Induced Apoptosis

The effects of APP on the mtPTP were quite dramatic in CHO cells. It is thought that inhibiting the mtPTP might be beneficial at reducing apoptosis. In fact we did observe a reduction of mtPTP activation and apoptosis with the supplementation of GSH, likely as a result of its antioxidant effects which would be
expected to de-sensitize the mtPTP to increases in intracellular calcium. The immunosuppressive drug cyclosporin A (CSA) has been shown to prevent depolarization of mitochondria and inhibit the mtPTP via binding to cyclophilin D, an integral component of the mtPTP [54]. In this preliminary experiment we transfected CHO cells for 6 h with APP and then treated the transfected cells with either 1μM or 10μM CSA to determine if direct inhibition of the mtPTP would be sufficient to protect against APP induced apoptosis. After imaging these cells for apoptosis and caspase-3 activation it became evident that our first experiment showed promising results in that cyclosporin A at the higher concentration was able to protect against apoptosis and caspase activation in APP overexpressing cells (Fig. 21).
Figure 21: Quantification of Apoptosis in Cyclosporin A Treated Cells. Images of apoptotic cells were observed for apoptosis, APP caused ~48% apoptosis. APP+CSA 1µM had ~39% apoptosis & 10µM CSA experienced 23% apoptosis. N=1.
CHAPTER FOUR: DISCUSSION

The two leading hypotheses of DS suggest that either the overexpression of a single gene is responsible for the characteristics of DS or that all the genes on the chromosome are no longer in homeostasis produce cognitive disturbances and neuronal death characterized in DS. Perhaps the overexpression of certain genes produces a deleterious effect downstream of the protein and gene product itself. Overexpression of genes such as APP have been shown to significantly increase the expression levels of Aβ, and it has been reported that increased serum levels of Aβ lead to aggregation and plaques formed by Aβ, in the Ts65Dn mouse reduction of amyloid beta serum levels has been shown to rescue memory and learning [63]. Although APP is the precursor protein for Aβ, it is the downstream effects of the protein that induces many of the cognitive hallmarks of AD and AD in DS.

The observations made in this study illustrate that overexpression of APP causes mitochondrial oxidative stress by depleting GSH, which consequently activates the intrinsic apoptosis cascade. These toxic effects of APP are significantly attenuated by compounds that target either the intrinsic apoptosis pathway (Bcl-2, Boc & Cyclosporin A) or oxidative stress (GSH). It has been reported that APP overexpression is able to induce increased intracellular levels of Ca²⁺, axonal pruning and general apoptosis. We have made the novel observation that as a result of causing mitochondrial oxidative stress, APP also induces the opening of mtPTP,
triggers release of cytochrome c from mitochondria, and activates the executioner protease, caspase-3 leading to apoptosis.

Our results show that APP causes apoptosis, this is consistent with previous studies showing that overexpression of APP triggers death in cells. Although the aforementioned studies suggested that APP acts primarily through an extrinsic pathway involving TNF receptors, we discovered that CHO cells die in response to APP through an intrinsic apoptotic pathway [38]. Caspase-3 is the executioner caspase and upon activation of this protease, the cell is committed to die by apoptosis. In APP overexpressing CHO cells caspase-3 activation was significantly increased indicating that the cells were overwhelmed by this protease and were induced to undergo cell suicide. Other studies have reported that the secretion of APP or Aβ is able to cause apoptosis via a paracrine mechanism in surrounding cells [64]. The previous report also suggested that the apoptosis induced by APP occurred through an extrinsic pathway. Conversely the death that CHO cells experience in response to APP occurs through an intrinsic mechanism. CHO cells that were transfected with APP were the only cells undergoing apoptosis, while cells that were not transfected remained healthy and viable. Thus, we found no evidence of a paracrine release mechanisms for APP toxicity in CHO cells.

Recent studies suggest that intracellular GSH depletion may result in mitochondrial malfunction [65]. The mitochondrial and cytosolic levels of GSH were significantly reduced in CHO cells overexpressing APP indicating that the functions of mitochondria may have been impaired. The loss of this essential
antioxidant due to the increased expression of APP indicates that these cells are succumbing to oxidative stress. Further study of the activation of the mitochondrial permeability transition pore demonstrated key involvement of mitochondrial oxidative stress is involved in activating this death pathway. The mtPTP is activated when components of the channel become oxidized (e.g., ANT). Thus if there is a loss of antioxidants in the mitochondria and APP increases oxidative stress, then susceptible mitochondria open the mtPTP allowing the intake of small molecules and water leading to outer membrane rupture and eventual cell death.

The result that APP failed to induce bax activation in our system was surprising given that bax is often implicated in the intrinsic apoptosis cascade. These findings lead us to believe that there may be another protein that is being activated in the intrinsic apoptosis cascade. Rises in intracellular Ca\(^{2+}\) have been reported to cause endoplasmic reticulum (ER) stress, which in turn results in a release of ER Ca\(^{2+}\) stores [66]. The Ca\(^{2+}\) released from the ER is then able to enter the mitochondria and once again activate the mtPTP and trigger intrinsic apoptosis. Observing the effects of APP overexpression on ER dynamics is of great importance due to the presence of an ER signaling sequence that APP carries [42]. The overexpression of APP may allow for this protein to insert itself into the membrane of the ER causing stress and general disruption of protein production causing degradation of the ER network and an eventual release of Ca\(^{2+}\) stores. The finding that bax is not involved in cell death due to APP overexpression also suggests that caspase-2 or an alternative caspase may be involved in the release of cytochrome c.
Caspase-2 is a direct activator of the intrinsic apoptosis pathway, and is also able to directly cause cytochrome c release [67]. Due to the negative results of active bax in APP expressing cells, further studies of alternative caspases and intrinsic apoptosis mediators such as Bid and Puma need to be performed.

Using the immunosuppressive drug cyclosporine A we were able to significantly prevent APP induced apoptosis. Cyclosporin A is known to prevent cyclophilin D from becoming a member of the mtPTP essentially inactivating the pore itself. The results that were observed when APP overexpressing cells were treated with cyclosporin A indicate that activation of the mtPTP is directly involved in the death pathway that our cells were experiencing. GSH and Bcl-2 were able to protect against the depletion of mitochondrial levels of glutathione as well as prevent caspase activation and the opening of the mtPTP. Through our mtPTP experiments we showed that inhibition of the pore mediates whether or not cells overexpressing APP undergo apoptosis unless blocked downstream by an alternative mechanism (eg. Boc).

During western blot analysis we noticed that CHO cells co-expressing APP and Bcl-2 produced a singlet band of protein of APP where as other treatments and APP alone repeatedly produced a doublet band of protein for APP. We hypothesize that Bcl-2 and a lower molecular weight peptide of APP are reacting thus the signal of the doublet was lost. Current studies support this hypothesis, Yang et al, found that overexpressed APP, Bcl-2 and certain heat shock proteins form a complex in mitochondria of stressed cells. Although this is a hypothesis, there is also
considerable data that suggest Bcl-2 can protect against amyloid-beta toxicity by lowering Ca^{2+} influx and prevent mitochondrial oxidative stress [68]. The mechanisms of how Bcl-2 is able to achieve these cellular protective effects are not well known, but further investigation could lead to discoveries that would explain the unexpected results that our APP western blots produced when co-expressing Bcl-2. In order to study the possible interactions of Bcl-2 and APP in our cell system we could perform a transfection and immunoprecipitation to determine whether or not APP or Bcl-2 are able to be pulled down together on a western blot. APP carries an ER localization signal, but it has also been shown to carry a mitochondrial localization signal which allows it to insert into the membrane of mitochondrial. The mitochondrial localization signal could be imperative to the Bcl-2 interaction due to Bcl-2’s key interactions with mitochondria and mitochondria import machinery.

Developing fetal brains are more susceptible to oxidative stress, due to the lack of a fully developed neural network that promotes the growth and retention of newly formed fragile neurons. The future directions of our experiments aim to replicate these results in primary neuronal cultures, and perhaps test these results in vivo on the widely used Ts65Dn DS mouse model. If these results can be replicated in neurons in vitro and in vivo in a mouse model of DS; mitochondrial oxidative stress, mtPTP activation, and the intrinsic apoptosis cascade may prove to be relevant therapeutic targets for the AD-like pathology observed in DS individuals.
APP and Aβ have proven to be toxic to cells in certain instances, in our study it was not determined that the toxicity CHO cells overexpressing APP were succumbing to was from APP itself or its cleavage product Aβ. In the future we would like to expand our study to measure Aβ peptide in DsRed-2 control cells and compare the levels to that of CHO cells overexpressing APP using an ELISA. Perhaps there is a certain threshold of APP production that causes a cell to undergo apoptosis or promotes a cell to produce more Aβ. Understanding the measureable levels of proteins produced either APP or Aβ would be beneficial to comprehend the range of toxic proteins that must be reduced to protect a cell from certain death. The way that these levels could be determined is by performing a titration of our plasmid and measuring apoptosis and protein levels in the media by an ELISA. Performing titrations would be essential to ensure that the levels of APP are comparable to that of in vivo levels.

The effects that DS and AD in DS produce are what mentally debilitate these individuals. Because DS occurs maternally in meiosis I, the ability to inhibit the triplication of HSA21 is beyond the reach of modern science. It is the treatment and reduction of the overexpression of the genes on HSA21 that is a more suitable way to treat individuals with DS and perhaps protect against the widespread neuronal loss that individuals with DS develop. Our results help to progress the field by identifying certain protective strategies to mitigate the toxicity induced by APP overexpression. These strategies may provide new therapeutic approaches to stem the progression of neuronal loss in DS and in AD in DS.
REFERENCES


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