Effects of Proteasome Inhibition on Tau Protein

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Institute of Medical Science
University of Toronto

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Abstract

The ubiquitin-proteasome system is an important proteolytic pathway implicated in the formation of neurofibrillary tangles from hyperphosphorylated tau, a pathological hallmark of tauopathies such as Alzheimer’s disease. We hypothesize that proteasome inhibition will result in tau accumulation, leading to the formation of aggregates. We established a combined pharmacological and genetic model of proteasome inhibition in SHSY-5Y neuroblastoma cells. Through lentiviral infection, we expressed a mutant T1A form of the β5 proteasome subunit to impair the chymotryptic proteolytic activity of the proteasome. Cells were then treated with different pharmacological inhibitors of the proteasome to further exacerbate the effects of mutation-mediated proteasome inhibition and tested for changes in tau expression. Contrary to our hypothesis, chronic proteasome inhibition and exposure to mild oxidative stress concurrently resulted in increased tau degradation. Therefore, although chronic proteasome inhibition was insufficient to induce changes in tau turnover, it rendered cells more vulnerable to oxidative insult.
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<tr>
<td>AD</td>
<td>Alzheimer’s Disease</td>
</tr>
<tr>
<td>Aβ</td>
<td>Amyloid beta</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle media</td>
</tr>
<tr>
<td>E1</td>
<td>Ubiquitin-activating enzyme</td>
</tr>
<tr>
<td>E2</td>
<td>Ubiquitin-conjugating enzyme</td>
</tr>
<tr>
<td>E3</td>
<td>Ubiquitin protein ligase</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>Hsc70</td>
<td>Heat shock cognate 70</td>
</tr>
<tr>
<td>M</td>
<td>Mock vector/ mock vector infected cells</td>
</tr>
<tr>
<td>NFT</td>
<td>Neurofibrillary tangles</td>
</tr>
<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
</tr>
<tr>
<td>PAC</td>
<td>Proteasome assembly chaperones</td>
</tr>
<tr>
<td>PS1</td>
<td>Presenlin 1</td>
</tr>
<tr>
<td>PS2</td>
<td>Presenlin 2</td>
</tr>
<tr>
<td>PSMβ2</td>
<td>Proteasome subunit β2</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>Rpn</td>
<td>Regulatory particle non-ATPases</td>
</tr>
<tr>
<td>Rpt</td>
<td>Regulatory particle triple A-ATPases</td>
</tr>
<tr>
<td>T1A</td>
<td>Threonine 1 to alanine</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris Buffered Saline with 0.5% TWEEN 20</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TetR</td>
<td>Tet repressor</td>
</tr>
<tr>
<td>TR-SH-SY5Y</td>
<td>SH-SY5Y neuroblastoma cells expressing the TetR protein</td>
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<tr>
<td>UBB</td>
<td>Polyubiquitin B</td>
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<tr>
<td>UCH-L1</td>
<td>Ubiquitin carboxyl terminal hydrolase L1</td>
</tr>
<tr>
<td>Ump1</td>
<td>Underpinning maturation of proteasome 1</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype/ Wildtype β5 subunit of the proteasome</td>
</tr>
<tr>
<td>Δtau</td>
<td>Tau species with the 20 amino acids cleaved from the carboxy-terminus</td>
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1 Introduction

1.1 Alzheimer’s Disease

Alzheimer’s disease (AD) is a progressive neurodegenerative disease that affects over 24 million people worldwide and the numbers are estimated to reach 80 million by 2040 (Ferri et al., 2005). Alzheimer’s disease accounts for 60-80% of all cases of dementia and has become the sixth leading cause of death in the United States (Alzheimer’s Association, 2011). About 95% of all AD cases are sporadic with age being the only common risk factor of the disease. The incidence of AD increases with age – 1 in 8 affected individuals are aged 65 and older and 43% of individuals develop AD by age 85 (Alzheimer’s Association, 2011). Unfortunately, despite the large amount of research effort into understanding AD, the mechanism of disease is still largely unclear and no cure is available for patients.

Alzheimer’s disease was first described in 1907 by a German neuropathologist, Dr. Alois Alzheimer. Initially, patients with AD experience short term memory loss, but this progresses to severe memory loss; attention, orientation, and language deficits; and inability to perform self-care functions (reviewed by Oddo, 2008). Pathologically, the brain develops severe atrophy and synaptic degeneration, with neuronal death in the limbic system and neocortex correlating with the level of cognitive impairment (reviewed by Bi, 2010). Alzheimer identified two classical hallmarks in the brains of AD patients, senile plaques composed mainly of amyloid β and neurofibrillary tangles composed largely of hyperphosphorylated tau, both of which are still used for diagnosis of the disease today.

1.1.1 Amyloid-β and Senile Plaques

Amyloid-β (Aβ) is a membrane protein whose physiological role is still largely unknown. It is the main component of senile plaques found in AD and is generated by sequential cleavage of the amyloid precursor protein (APP) by β and γ-secretase. APP can also be cleaved by α secretase, which prevents the formation of Aβ as its cleavage site lies within the Aβ domain.
Amyloid β is commonly found in two isoforms, Aβ40 and Aβ42, which are 40 and 42 amino acids in length, respectively. Although both isoforms have been found to be involved in AD, Aβ42 is considered more cytotoxic than Aβ40 as it is more prone to aggregation at intracellular pH, is less susceptible to degradation, is more hydrophobic, and accumulates more readily in endosomes and lysosomes (reviewed by Glabe, 2001). Insoluble aggregates that are highly resistant to degradation within cells are often made up of the Aβ42 isoform, which suggests that the pathology and cytotoxicity of Aβ may be largely in due to this isoform. Accumulation of intracellular Aβ has been found to induce toxicity within the cells, prior to the formation of senile plaques in mice (McGowan et al., 2005) and in cell and primary neuron cultures (reviewed by Li et al., 2007), suggesting an innate toxicity of Aβ preceding protein aggregation. Amyloid β can exist in the form of monomers, dimers, or can go on to form cytotoxic extracellular fibril deposits and senile plaques as seen in AD (Selkoe, 2001). Mutations in the APP gene, presenilin 1 (PS1), and presenilin 2 (PS2), have been associated with familial AD, which accounts for 5% of all AD cases (reviewed by Bi, 2010). PS1 and PS2 are both involved in the proper expression and activity of γ-secretase, and mutations in these genes alter the production ratio of Aβ isoforms Aβ40 and Aβ42 (reviewed by Pimplikar, 2009). As mentioned before, Aβ42 is more prone to aggregation and an increased Aβ42:40 ratio has been found to correlate with the age of AD onset (Bentahir et al, 2006). Mutations in these three genes have been shown to increase Aβ production and aggregation (Selkoe, 2001). This supports a strong role of Aβ in familial AD, although its role in sporadic AD still remains to be confirmed as the level of amyloid plaque accumulation does not correlate with the severity of dementia in AD patients (Terry et al., 1991). Also, it has been reported that there is amyloid deposition in aged individuals with no impairment in cognition (Aizenstein et al., 2008), proposing that Aβ deposition is not sufficient on its own to induce AD. More recent studies have shown a correlation between amyloid levels in the brain with the severity of cognitive decline, although this association was not specific to those with AD as those who did not develop dementia displayed a similar decline in cognition ability with high amyloid levels in the brain (Cosentino et al., 2010).
1.1.2 Tau Protein and Neurofibrillary Tangles

Tau is a microtubule-associated protein that is involved in microtubule assembly and stabilization, and has roles in axonal transport as well (Roy et al., 2005). The six major isoforms of tau within the central nervous system result from alternative splicing of exons 2, 3, and 10 of a single gene. The isoforms can be distinguished based on two domains: the amino-terminal projection domain and the carboxy-terminal microtubule-binding domain. The projection domain is composed of an acidic-rich region while the microtubule binding domain contains highly conserved tubulin-binding motifs. The isoforms contain either three or four tubulin-binding repeats (3R and 4R, respectively) at the carboxyl terminal and may contain zero, one or two 29 amino-acid inserts within the projection domain (0N, 1N, and 2N respectively), with the two domains separated by a proline-rich basic domain (Figure 1.1).
Figure 1.1: Six isoforms of tau within the human central nervous system. 3R and 4R refers to 3 and 4 tubulin-binding repeats (shown in red), respectively and N represents the number of 29 amino-acid inserts at the N-terminal portion of the protein (shown in yellow).

The projection domain is responsible for the spatial distribution of axonal microtubules, and interactions with cytoskeletal proteins and other proteins (reviewed by Avila et al., 2004). The microtubule binding domain plays a role in promoting microtubule stability. The proline-rich regions of tau bind to the outer surface of assembled microtubules, but in the presence of tubulin prior to microtubule assembly, tau binds to specific pockets within β-tubulin on the inside of microtubules instead (reviewed by Avila et al., 2004 & Ballatore et al., 2007). The cycling of tau on and off of microtubules maintains microtubule integrity and allows for axonal transport of cargo down axons. The level of microtubule stability differs between 3R and 4R tau isoforms. 4R tau binds with greater affinity and strength to microtubules and can displace 3R tau previously associated with microtubules (Lu & Kosik, 2001). In normal adult brain, the 3R and
4R isoforms are expressed in equal proportion in most areas of the brain and deviations can result in neurodegenerative diseases such as frontotemporal dementia and parkinsonism linked to chromosome 17 (Hong et al., 1998; Varani et al., 1999).

Although tau can undergo many types of post-translational modifications such as glycation, ubiquitylation, nitration and glycosylation (reviewed by Ballatore et al., 2007), phosphorylation is considered to be the most notable modification contributing to the microtubule-binding ability of tau. The longest form of adult brain tau is estimated to have over 80 phosphorylation sites (Goedert et al., 1989) with over 39 phosphorylation sites identified to be phosphorylated in paired helical tau (Hanger et al., 2007). In all tauopathies known to date, tau has been abnormally hyperphosphorylated (Lee et al., 2001). Hyperphosphorylation of tau promotes its dissociation from microtubules, increasing its levels within the cytosolic pool. Abnormal accumulation of cytosolic tau encourages misfolding and association with membranes, forming small nonfibrillary deposits of tau or pretangles (Mena et al., 1996). Conformational changes, such as β-sheet formation, in pretangles result in the subsequent assembly of straight and paired helical filaments and neurofibrillary tangles (NFT) (reviewed by Kuret, 2005), as seen in AD. Davies and colleagues (1999) found that phosphorylation of tau by protein kinase A occurs on two sites, Ser\(^{214}\) and Ser\(^{409}\). By harvesting specific antibodies to recognize these two phospho-tau epitopes, they were able to show staining in the brains of AD patients and not in controls. Phosphorylation of tau at Ser\(^{214}\) and Ser\(^{409}\) was found to occur prior or concurrently with the initial formation and detection of tau aggregates. This further supports the role of phosphorylation on tau aggregate formation in AD.

The level of NFT within the brain correlates with the level of cognitive impairment in those suffering from AD, which contrasts with the relationship between levels of senile plaques and cognition mentioned previously (Arriagada et al., 1992; Santacruz et al., 2005; Oddo et al., 2006). This supports the notion that NFT plays a direct role in the progression of AD as NFTs not only act as a physical obstruction to axonal transport signalling proteins within neurons, they also promote the sequestration of more tau and proteins into aggregates, resulting in a further loss of tau function and the functions of any other accumulated proteins. The formation of NFTs has also been shown to induce synaptic loss and neuroinflammation (reviewed by Ballatore, 2007). Preceding the formation of NFT, the presence of pre-aggregated forms of tau has also been
shown to be toxic in vitro (Khlistunova et al., 2006).

Proteolysis and truncation of tau seem to play an important role in NFT formation and AD disease progression. A specific tau species with the 20 amino acids cleaved from the carboxy-terminus (Δtau) has been found in NFTs in AD patient brains (Gamblin et al., 2003). It has also been suggested that truncated tau can promote faster polymerization into fibrils, as well as exert innate cytotoxic effects (reviewed by García-Sierra et al., 2008). Phosphorylation of tau at Ser\textsuperscript{422}, the cleavage site for the formation of Δtau, occurs prior to the cleavage event (Guillozet-Bongaarts, et al., 2006) and co-transfection of Δtau with glycogen synthase kinase 3 (GSK3), a common kinase for tau, leads to the formation of thioflavin-S positive Δtau aggregates. Both these lines of evidence suggest that phosphorylation of tau not only promotes the dissociation of tau from microtubules at the earlier stages of tau pathology but also contribute to the formation of specific tau species prone to NFT formation in the later stages. Therefore, modifications of tau may play different roles at different stages of AD progression.

1.1.3 Amyloid Cascade Hypothesis

The amyloid cascade hypothesis originally suggested that the accumulation of Aβ is the cause and driving factor of AD. Two Aβ isoforms, Aβ40 and Aβ42, are involved in the formation of cytotoxic senile plaques (Hardy and Higgins, 1992; Walker et al., 2000), which initiates downstream events such as hyperphosphorylation of tau, synaptic loss, and neuroinflammation, all of which contribute to AD pathology (reviewed by Pimplikar, 2009). Patients with familial AD also carry mutations in genes related to Aβ formation and accumulation. However, it was later found that not only did the level of Aβ not correlate with the level of dementia in patients (Terry et al., 1991; Aizenstein et al., 2008), Aβ plaques can be found in people without any signs of AD or cognitive impairment (Villemagne et al., 2008). Therefore, the current view suggests that Aβ is likely not an upstream event but occurs simultaneously to all the other factors acting on AD progression (reviewed by Pimplikar, 2009). It has been shown in a transgenic AD mouse model that Aβ and tau synergistically contribute to mitochondrial dysfunction, which plays a role in neurodegeneration, with Aβ and tau targeting different steps within the respiratory
cycle (Rhein et al., 2010). Interestingly, it has been shown that in the absence of tau, APP23 transgenic mice were protected from Aβ-mediated cytotoxicity (Ittner et al., 2010). Knockout of tau was also able to rescue memory impairments and neuronal cell death in mutant APP/PS1 transgenic mice (Brion et al., 2010). Vossel and colleagues (2010) were also able to demonstrate that a reduction in tau prevented Aβ-induced impairments in axonal transport in cultured neurons and prevented Aβ-mediated neuronal and behavioural impairments in AD transgenic mice. All recent evidence seems to suggest that tau may a critical role in the development and progression of AD.

1.2 Autophagy-Lysosome Degradation Pathway

To maintain physiological homeostasis, misfolded or unnecessary proteins are mainly degraded by one of two pathways: the autophagy-lysosome pathway or the ubiquitin-proteasome system. Autophagy is the process by which damaged or unwanted proteins are delivered toward lysosomes for degradation and is generally considered a non-discriminative process that degrades long-lived proteins in bulk. This degradation pathway has been implicated in the elimination of protein aggregates as seen in Alzheimer’s disease. Autophagy can be generally classified into three separate pathways based on the method by which protein substrates are delivered toward the lysosome for degradation: macroautophagy, microautophagy, and chaperone-mediated autophagy (Figure 1.2).
Macroautophagy is the most common and elaborately studied form of autophagy. Double-membraned vacuoles known as autophagosomes engulf unwanted cellular debris, including entire organelles, into enclosed vacuoles. The outer membrane of autophagosomes fuses with lysosomes to deliver the autophagic body into the lysosomal vacuole for degradation. This pathway has been found to act as a survival mechanism in response to a cell’s exposure to environmental stresses such as nutrient deprivation (reviewed by Kiel, 2010)

Microautophagy involves the invagination and subsequent budding of the lysosomal vacuoles to form single membrane vesicles within lysosomes to contain proteins deemed for degradation. Similar to macroautophagy, microautophagy can process proteins as well as entire organelles for degradation. The role and regulation of microautophagy still remains to be determined although evidence suggests that this process is dependent on GTP and calcium (Uttenweiler & Mayer, 2008).
Lastly, chaperone-mediated autophagy differs from the other two pathways as it is a more selective method of autophagy and is unable to degrade entire organelles (Kaushik et al., 2008). Chaperone-mediated autophagy involves the recognition of a KRERQ sequence motif in proteins specifically targeted for lysosomal degradation by chaperones such as heat shock cognate 70 (Hsc70). This autophagic pathway works together with macroautophagy in response to cell starvation. If nutrient deprivation persists and the macroautophagy pathway becomes overwhelmed, chaperone-mediated autophagy is activated to degrade non-essential proteins for recycling of amino acids necessary for replenishing essential proteins within the cell (Fuertes et al., 2003; Massey et al., 2006). Cellular stress induces the unfolding of proteins to reveal the KRERQ sequence that is normally inaccessible when the protein in its normal conformation. The selectivity of chaperone-mediated autophagy in a stressed cell also prevents the degradation of healthy and necessary proteins in close proximity of damaged ones.

1.2.1 Autophagy and Alzheimer’s Disease

Aside from a role of autophagy in maintaining basal homeostasis in cells, autophagy has also been found to play a role in neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease and frontotemporal dementia (reviewed by Funderburk et al., 2010). The literature mostly supports a neuroprotective role of autophagy in neurodegenerative diseases by assisting in protein aggregate clearance within neurons. For example, inhibition of the autophagy process was found to induce an accumulation of ubiquitinated proteins in the form of inclusions within neural cells (Hara et al., 2006). Induction of macroautophagy by rapamycin treatment was also found to reduce insoluble tau levels and toxicity induced by expression of mutant R406W tau in Drosophila (Berger et al., 2006) while inhibition of the lysosomal system by chloroquine or 3-methyladenine inhibited tau degradation and promoted aggregate formation (Hamano et al., 2008). Tau transgenic animal models have also displayed signs of impaired autophagic-lysosomal activity whereby autophagosomes and lysosomal complexes displayed an accumulation of filamentous tau aggregates (Lim et al., 2001; Lin et al., 2003).

It has been speculated that dysfunction of the autophagy-lysosome pathway may be a result of
tau hyperphosphorylation in Alzheimer’s disease. Tau is involved in axonal transport of cargo throughout the cell and hyperphosphorylation of tau promotes the dissociation of tau from microtubules, resulting in axonal network instability within the cell. This impairs the trafficking of autophagic vesicles within neurons and results in a build up of autophagosomes and unwanted proteins within the cell (Nixon et al., 2005; Yue, 2007).

However, some studies have suggested that induction of macroautophagic activity may actually contribute to Alzheimer’s disease pathology as increased lysosomal activity and expression of lysosomal enzymes such as cathepsins have been found to induce Aβ formation (Cataldo et al., 1995; Chevallier et al., 1997). Macroautophagy was also found to be activated in animal models of Alzheimer’s disease prior to the deposit of protein aggregates and contributed to the generation of Aβ (Yu et al., 2005). Reducing autophagic activity in Drosophila was found to be protective against Aβ induced toxicity, while induction of autophagy by rapamycin induced neurodegeneration and toxicity (Ling et al., 2009).

Therefore, it seems autophagy plays a role in promoting tau degradation but on the contrary, also promote the formation and accumulation of Aβ within neurons and so, it remains to be determined if autophagy is a neuroprotective or neurotoxic mechanism in Alzheimer’s disease.

1.3 Ubiquitin Proteasome System

The ubiquitin proteasome system is the major proteolytic machinery in eukaryotic cells. It is responsible for the turnover of 80-90% of all intracellular proteins and disruption in its proper function results in an accumulation of unwanted and damaged proteins within the cell. Protein substrates targeted for degradation by the 2000 kDa 26S proteasome are tagged with a polyubiquitin chain consisting of four or more ubiquitin monomers. One or two ATP-dependent 19S regulatory units can bind to either ends of a 20S catalytic core, forming the 26S proteasome complex, and are responsible for the recognition and binding of ubiquitinated protein targets (Figure 1.3). The 20S catalytic core can also bind to PA28 activator complexes or be found without any regulatory particles at all.
Figure 1.3: The Ubiquitin-Proteasome System

1.3.1 Ubiquitin

A highly conserved 76 amino acid residue protein in eukaryotes, ubiquitin acts primarily as a signalling protein when attached to protein substrates. Ubiquitination at different levels achieves different signalling roles within the cell. Monoubiquitination to histones was found to play a role in gene silencing in yeast, as well as affect other post-translational changes that mediate transcription (reviewed by Muratani & Tansey, 2003). Multiubiquitination, or conjugation of multiple monoubiquitins, has roles in endocytosis and lysosomal degradation (reviewed by Haglund et al., 2003). In regards to signalling within the ubiquitin proteasome system, most proteasome substrates are tagged with a polyubiquitin chain. Each ubiquitin molecule contains 7 lysine residues that can all participate in formation of the ubiquitin chain, although Lys48 and Lys63 is the most common ligation site (Varadan et al., 2002 & 2004). Polyubiquitin chains
formed at Lys48 is of particular relevance to proteasome degradation signalling (Thrower et al., 2000; Richly et al., 2005) while Lys64 chains are mostly found in endocytosis and protein kinase activation pathways (Wu et al., 2006).

The attachment of the polyubiquitin chain to target proteins occurs in a sequence of reactions involving the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and a group of ubiquitin protein ligases (E3) (Figure 1.4). When a damaged or unwanted protein is identified for degradation by the proteasome, E1 activates an ubiquitin protein in an ATP-dependent process. Ubiquitin-conjugating enzymes then transfer the activated ubiquitin to an E3, which ligates the ubiquitin monomer to the target protein. This process is repeated until 4 monomers or more have been transferred to form a polyubiquitin chain. The 19S particle recognizes the polyubiquitin chain and processes it for degradation through the 20S proteasome. After proteolysis through the proteasome, deubiquitinating enzymes, such as ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), cleave the ubiquitin separated from the target protein back into ubiquitin monomers for recycled use within the cell.

Figure 1.4: Ubiquitination Pathway.
1.3.2. 19S Regulatory Particle

The 19S regulatory particle is made up of 6 homologous Regulatory particle triple A-ATPases (Rpt) and at least 11 Regulatory particle non-ATPases (Rpn). The base of the 19S particle is made up of the 6 Rpt subunits and Rpn1, 2, and 10 while the lid is formed by Rpn3, 5, 6, 7, 8, 9, 11, and 12, with the possible addition of other Rpn subunits (reviewed by Voges et al., 1999; Sorokin et al., 2009). The assembly of the 19S particle is still under debate. There is no clear evidence stating whether the 19S regulatory particle is assembled prior to attaching to the 20S catalytic core, or whether the 19S particle is assembled on the 20S unit, with evidence existing in support of both notions. The optional addition of a number of Rpn subunits also contributes to the difficulty of deciphering the actual assembly process of the 19S particle.

The proteasome is able to perform selective degradation of damaged or unwanted proteins within the cell due to its ability to recognize a specific polyubiquitin chain that targets tagged proteins to the proteasome for proteolysis. This polyubiquitin chain, consisting of four or more ubiquitin monomers, is recognized by the ATP-dependent 19S particle. Upon recognition of the polyubiquitin chain by the lid of the 19S particle, conformational changes occur that initiate the opening of the 20S catalytic core to allow proteins to translocate through the core for degradation (Strickland et al., 2000). The base of the 19S particle then unfolds and orients the target substrate through the 20S core for degradation. After degradation through the catalytic core, the 19S regulatory lid contains deubiquitinating enzymes to hydrolyse the bond between ubiquitin and the target peptide, recycling the ubiquitin back into the cell (Verma et al., 2002; Guterman & Glickman, 2004). The recognition, translocation and deubiquitination steps of proteasome degradation are all ATP-dependent processes.

1.3.3 PA28 Activator Complex

Aside from the 19S regulatory complex, the 20S catalytic core can also bind to the PA28 activator complex in mammalian cells, but not in yeast. The PA28 complex is a heptameric ring composed of PA28α and PA28β subunits. The PA28-20S proteasome complex performs a
similar role to 19S regulatory particles but in an ATP- and ubiquitin-independent pathway and it mainly degrades small peptides that do not require extensive unfolding prior to translocation through the 20S catalytic core.

1.3.4 20S Catalytic Core

The 20S proteasome is the catalytic core responsible for the multi-proteolytic function of the proteasome system. In eukaryotes, it is composed of 28 subunits: 7 different α-subunits and 7 different β subunits in duplicate. The α and β subunits form two heptamer rings, with the two α rings sandwiching the two β rings (α₁β₁β₂α₂) in a stacked column to form the 20S proteasome (Figure 1.3). The 20S proteasome is composed of three interconnected compartments: an internal proteolytic cavity (β₁β₂) between two antechambers (α₁β₁ and β₂α₂).

The antechambers act as reservoirs for proteins prior to degradation and play important roles in unfolding and maintaining the proper configuration of proteins targeted for degradation through the proteolytic cavity (Ruschak et al., 2010). The α ring is also responsible for the gating of the 20S proteasome as α subunits contain a N-terminal α-helix which points inward toward the centre of the chamber, blocking off access to the proteolytic cavity. Upon activation of the proteasome by substrate binding, allosteric changes in the α ring expose a gate opening about 13Å in diameter which suggests that only small or unfolded peptides can translocate through the core for degradation. The α₃ subunit seems to play the most prominent role in gate opening as its N-terminal forms contacts with every other α subunit within the ring and stabilizes the conformation of the α ring (Groll et al., 1997 & 2000; Koehler et al., 2001).

The internal proteolytic cavity is formed by the two β rings within the 20S proteasome. Of the seven distinct β subunits, only three have established proteolytic activities. The β₁ subunit has caspase-like activity and cleaves after acidic and negatively-charged amino acid residues. The β₂ subunit possesses tryptic activity and hydrolyzes after basic and positively-charged amino acid residues. Lastly, the β₅ subunit displays chymotryptic activity and cleaves after hydrophobic residues. Three additional catalytic subunits, β₁i, β₂i, and β₅i, are synthesized to
replace the constitutive β1, 2 and 5 subunits in response to stimulation by γ-interferon during an immune reaction, forming immunoproteasomes (reviewed by Sorokin et al., 2009).

The correct assembly of α subunits into α rings is mediated by proteasome assembly chaperones (PAC) complexes within the cell. PAC heterodimeric chaperone complexes assist in the correct assembly of α subunits by promoting proper α subunit ligation and proof-reading the arrangements of the subunits to ensure proper assembly (reviewed by Gallastegui & Groll, 2010). β subunits cannot incorporate into β rings without the preassembly of α rings. After formation of the ring, β subunits are sequentially attached in three steps: firstly by attaching subunits β2, β3, and β4; then subunits β1, β5, and β6; and finally β7 to complete the formation of a half proteasome. With the help of maturation factor Ump1 (underpinning maturation of proteasome 1), the C terminus of the β7 of a half proteasome reaches out to the β1 and β2 subunits of the coupling half proteasome, to form and stabilize a full pre-proteasome (Ramos et al., 2004) (Figure 1.5).

![Figure 1.5: Assembly of α and β proteasome subunits (blue and green, respectively) into full 20S proteasome.](image)

The β7 subunit is also important for promoting the maturation and activity of the β1 subunit after assembly into the pre-proteasome (Ramos et al., 2004). β subunits are assembled into pre-proteasomes as propeptides and only after cleavage of the propeptides does the pre-proteasome become proteolytically active 20S proteasomes. The maturation of the proteolytic β subunits 1, 2,
and 5 reveals the N-terminal amino group required for nucleophilic attack of target proteins through the catalytic core. The prosequences do not seem to play a vital role in the assembly or positioning of the β subunits, but act more like chaperones in increasing the efficiency of proteasome assembly by promoting proper folding (Schmidt et al., 1999), although evidence for more crucial roles for the prosequences can be seen in higher organisms (Schmidtke et al., 1996; Kingsbury et al., 2000).

The processing of the β subunit propeptides into mature β subunits is achieved by a two step autocatalysis process (Schmidtke et al., 1996). The propeptide possesses two cleavage sites, one within the prosequence, and one at the consensus site. The cleavage within the prosequence helps unify the prosequence lengths for subsequent autocatalysis at the consensus site to form mature β subunits. After processing of the β subunit propeptides, the β subunits undergo conformational changes for full maturation (reviewed by Voges et al., 1999), yielding a fully mature and proteolytically active proteasome. The maturation chaperone Ump1 is then degraded by the proteasome upon successful assembly and maturation of the proteasome.

In our study, we take advantage of this maturation process to generate a genetic mutation of the β5 subunit to specifically inhibit the chymotryptic activity of the proteasome. By introducing a mutation at one of two cleavage site of the β5 subunit propeptides, the full maturation of the β subunit is interrupted. This allows for inhibition of the chymotryptic activity of the proteasome without disrupting the assembly process of the mutant β5 subunit with endogenous proteasome subunits into full proteasome complexes.

### 1.3.5 Proteolytic Degradation by the Proteasome

The 20S proteasome degrades target proteins in a processive manner, without the release of protein intermediates until the termination of the degradation process. The internal proteolytic chamber traps proteins as they are degraded into the desired product length then releases the degraded peptide chains (reviewed by Voges et al., 1999). Target proteins are typically cleaved to peptide chains 8 residues long on average, although they can range from 4 to 25 residues (Kisselev et al., 1998). The exact mechanism which determines product sizes are still under
debate and requiring further investigation.

1.3.6 Inhibition of the Proteasome by Pharmacological Agents

Researchers have made use of a wide variety of pharmacological agents in studies related to the proteasome. Proteasome inhibitors mostly target the chymotryptic activity of the proteasome, although they have also been shown to also affect the tryptic and caspase-like activities at higher concentrations. These inhibitors can be generally divided into two groups: synthetic and natural.

Since the proteolytic activity of the proteasome depends largely on the hydroxyl group on the threonine residue of β1, β2, and β5 subunits, synthetic peptide aldehydes that readily react with the hydroxyl group was the first major group of synthetic proteasome inhibitors used. Common peptide aldehydes include MG132 and PSI. These compounds inhibit the proteasome by forming a reversible covalent bond with the hydroxyl group and preventing the interaction between target proteins with the proteolytic site of the proteasome. Although they are potent inhibitors of the proteasome, they have also been found to interact with other serine and cysteine proteases such as cathepsin B and calpain. Researchers have attempted to develop more specific alternatives but new analogs still possess a number of limitations such as inducing apoptosis with prolonged treatment (reviewed by Myung et al., 2001).

Natural inhibitors tend to be more specific and potent, with lactacystin and epoxomicin being the most commonly used. Lactacystin forms an irreversible bond with the β subunit hydroxyl group to form an ester while epoxomicin interacts with the hydroxyl group of the β subunit threonine, then forms an irreversible complex with the resulting free amino group (Kisselev & Goldberg, 2001). Epoxomicin is a more specific proteasome inhibitor with no other known targets within the cell while lactacystin has been found to interact with other cellular proteases (Kozlowski et al., 2001).

Efforts have been done in search of more specific proteasome inhibitors that can target each of the three proteasome activities separately for researchers investigating the proteasome.
The use of proteasome inhibitors should be done with caution as treatment with proteasome inhibitors can result in the induction of apoptosis and formation of aggresomes in a number of different cell lines (reviewed by Wojcik & Demartino et al., 2003). Aggresomes are aggregates composed of proteasomes and ubiquitinated proteins that are formed when a high level of proteins targeted for proteasome degradation accumulate within the cell (Wojcik et al., 1996; Wojcik, 1997). Aggresomes are located in specific domains within the cell often referred to as “proteolysis centers”, which are specific regions within cells that have a high level of proteasome degradation activity. Aggresomes have also been found to accumulate at the microtubule organizing center (MTOC) of the cell in response to treatment with proteasome inhibitors (Anton et al., 1999). Aggregates may be localized to the MTOC to facilitate its degradation by proteasomes or by autophagy. Aggresome formation has also been speculated to occur in a number of different neurodegenerative diseases (Sherman & Goldberg, 2001). Therefore, the use of proteasome inhibitors may not only inhibit the proteolytic activity of the proteasome, but also induce other physiological events within the cell that contributes to the formation of aggregates and signs of neurodegeneration.

1.4 Ubiquitin Proteasome System Impairment and Age

A number of neurodegenerative diseases, such as Alzheimer’s and Parkinson’s disease, show increased risk with increased age, which correlates with the increasing impairment of proteasome function with age. It is speculated that this impairment of proteasome function with age is in part due to the accumulation of oxidized proteins and increased oxidative stress with age, although some also speculate that the converse, a decrease in proteasome proteolytic activity results in an accumulation of oxidized proteins (Ding et al., 2006; Cecarini et al., 2007), may be true. The proteasome is involved in the degradation of oxidized proteins, along with ubiquitinated proteins, within the cell. With age, there is decreased proteasome subunit expression, modifications in subunit expression and decreased proteasome activity (reviewed by Huang & Figueiredo-Pereira, 2010), which may all contribute to decreased proteolysis of unwanted proteins within the cell.
1.4.1 Oxidative Stress and the Proteasome

The major proteolytic pathway for the degradation of oxidized proteins is through the 20S proteasome system (Breusing & Grune, 2008). The effects of oxidative stress on the proteasome seem to differ depending on the level of oxidation within the cell. Mild oxidative stress has been found to potentiate the level of proteasome activity with the cell. This is likely due to an increased affinity for protein degradation of proteins mildly unfolded due to mild oxidative stress. Unfolded proteins gain access to the proteolytic chamber of the proteasome with greater ease, thereby increasing the rate of degradation. There may also be an increase in proteasomal activity in response to environmental stresses (Bossola et al., 2003), in an effort to bring the cell back to homeostasis.

Degradation of oxidized proteins most likely goes through a ubiquitin-independent 20S proteasome pathway of proteasome degradation. The 20S proteasome was found to be more resistant to oxidative stress compared to the 26S proteasome (Reinheckel et al., 1998 & 2000). Oxidized proteins were also found to be degraded in an ATP- and ubiquitin-independent manner (Shringarpure et al., 2003). Under exposure to environmental stressors that cause oxidative stress, proteolysis through the 20S pathway would help increase the rate of protein degradation by bypassing the time- and energy-consuming step of ubiquitinating the target proteins. This would help prevent the accumulation of oxidized proteins and prevent further crosslinking and aggregation of the damaged proteins and also release protein peptides that can be readily recycled for the translation of new proteins within the cell.

However, when the level of oxidative stress surpasses the cell’s ability to cope, which is a normal occurrence in aging, oxidation induces covalent crosslink and disulfide bond formation, which promotes the formation of aggregates (Davies, 2001) that can act as a physical obstruction to the proteasome catalytic core, thereby impairing proper proteasome function. Increasing exposure to oxidative stress in aging individuals likely contributes to a decrease in proteasome activity as reactive oxygen species have been shown to modify and inhibit the proteasome (reviewed by Ding & Keller, 2003). Impairment of the proteasome also impairs the cell’s ability to respond to environmental stresses, which further contributes to the detrimental effects of oxidative stress with age. Exposure to oxidative stress may also impair enzymatic activities that
assist the degradation of proteins within the cell. For example, Ubiquitin carboxyl terminal hydrolase L1 (UCH-L1) is an enzyme that helps break down polyubiquitin chains into usable monoubiquitin peptides for recycled use within the cell. However, its hydrolase activity is decreased when exposed to oxidative stress (Choi et al, 2004). This depletes the levels of available ubiquitin for ubiquitin-mediated degradation of damaged proteins through the 26S proteasome pathway. Butterfield and Boyd-Kimball (2004) found that the impairment of UCH-L1 correlates with an increase in protein accumulation and a decrease in proteasome activity.

Taking all these factors into consideration, increasing exposure to oxidative stress during aging results in several factors that may contribute to neurodegeneration: 1) the accumulation of protein aggregates due to the formation of covalent bonds, 2) the inhibition of proteasome activity by the presence of such aggregates, 3) a decrease in the cell’s ability to respond to environmental stress due to the impairment of proteasome activity and accumulation of protein aggregates overwhelming the cell, and 4) a loss of chaperone and enzymatic activities that help mediate proteasome degradation.

In addition, although proteasomes are ubiquitously found in both the nucleus and cytoplasm, exposure to environmental stress localizes proteasomes to lysosomes, likely due to autophagic degradation of damaged surrounding proteins (reviewed by Wojcik & DeMartino, 2003). The acidic environment of lysosomes renders proteasomes inactive resulting in an inhibition of proteasome activity and the presence of lysosomal enzymes proceed to degrade the proteasome complex as well.

1.4.2 Ubiquitin Proteasome System and Alzheimer’s Disease

A large number of neurodegenerative diseases display signs of toxic protein aggregation and accumulation, which impact cell viability and function. Since the ubiquitin proteasome system is involved in 80-90% of normal protein degradation within the cell, its impairment has been implicated in the dysregulation of proper protein homeostasis as seen in neurodegenerative diseases. Inhibition of the proteasome would result in the accumulation of protein aggregates that impair proper cell signalling – signalling proteins within the cell that are not being properly degraded lead to nonspecific signalling and build up of other proteins act as a physical
obstruction to cell signalling pathways. Neurons that become disconnected from the central signalling network result in cell death. Neurons within the central nervous system are especially vulnerable to the detrimental effects of proteasome inhibition as they are postmitotic and are unable to disperse damaged proteins within the cell through mitosis, leading to a sustained accumulation (Grune et al, 1997). This could render the central nervous system neurons more susceptible to protein accumulation and aggregation that lead to proteasome inhibition and vice versa.

In vitro studies have shown that inhibition of the proteasome is sufficient to induce neuronal cell death in vulnerable cell types by inducing apoptosis (reviewed by Ding & Keller, 2006). Major chronic neurodegenerative diseases such as AD, Parkinson’s disease, and amyotrophic lateral sclerosis all present with impaired proteasome activity and abnormal accumulation of ubiquitinated proteins in the form of inclusions (reviewed by Mayer, 2003), suggesting an impairment of the ubiquitin proteasome system to degrade ubiquitin-tagged damaged proteins. Paired helical filament tau has been co-immunoprecipitated with proteasomes isolated from patients with AD (Keck et al, 2003), suggesting a role of the proteasome in the turnover of paired helical filament tau in AD patients. Under physiological conditions, ubiquitination of tau has been found to signal tau for proteasome degradation (Liu et al, 2009). The ubiquitination of tau has been speculated to occur after hyperphosphorylation of tau, prior to the formation of NFT (Necula & Kuret, 2004). The literature supports the idea that phosphorylation of tau, along with other posttranslational modifications of tau such as glycation and nitration, reduces the degradation and increases the aggregation of tau into NFT which is then further ubiquitinated (reviewed by Martin et al., 2011). The accumulation of tau is correlated with the level of ubiquitination (Riederer et al., 2009), implicating that ubiquitination may prevent the proper degradation of tau inclusions within neurons, as seen in patients with AD. Proteasome activity was shown to be preferentially inhibited in the vulnerable brain regions that demonstrate high levels of oxidative stress, aggregate formation and neurotoxicity (reviewed by Ding & Keller, 2003).

The extent of age-related decline in proteasome activity differs with organism, tissue and mitotic state, but specifically in relation to neurodegeneration, there is an age-related decrease in chymotryptic activity throughout the central nervous system in rats (Keller et al., 2000).
humans, Keller and colleagues (2000) found significant impairment of chymotryptic and caspase-like activity in patients of AD, with effects most prominent in the hippocampal region. They concluded that the decrease in proteasome activity was not due to decreased expression of proteasome subunits, and the decline is most likely attributed to posttranslational modifications such as oxidative stress and accumulation of protein aggregates. Enzymes that help promote proteasome function, such as UCH-L1, were also found to be down-regulated in patients with AD (Castegna et al, 2002; Butterfield & Kimball, 2004).

1.4.3 Genetic Factors Impairing Proteasome Function in Alzheimer’s Disease

In concert with post-translational modifications that impair proteasome activity, patients with neurodegenerative diseases also display genetic factors that contribute to impairment of proteasome activity.

Polyubiquitin B gene is one of three genes encoding for ubiquitin in humans. A mutant form of polyubiquitin B, UBB$^{+1}$, was detected in patients with AD and other neurodegenerative diseases (reviewed by van Leeuwen et al., 2006). This mutation results in ubiquitin molecules that lack a Gly$^{76}$ at the C-terminus, which prevents it from binding to target proteins. UBB$^{+1}$ is still able to form polyubiquitin chains but chains capped with UBB$^{+1}$ are not readily broken down by deubiquitinating enzymes and greatly retard the proteasome degradation pathway (Lam et al., 2000). At high concentrations, it is able to inhibit the ubiquitin proteasome system and promote AD pathogenesis (De Vrij et al., 2001).

As mentioned before, UCH-L1 is a deubiquitinating enzyme required for dissembling the polyubiquitin chain used for protein targeting to the proteasome. Mutation in the gene encoding for UCH-L1, uch-ll, is considered a major contributor to AD (Choi et al., 2004) as UCH-L1 is not only involved in the proteasome degradation pathway of unwanted proteins within the cell, but it also plays important roles in synaptic signalling and is essential in long-term memory (Hedge et al., 1997; Gong et al., 2006). Reintroduction of UCH-L1 in AD transgenic mice was
able to restore deficits in synaptic signalling due to Aβ. Therefore, mutations in the uch-11 gene may play an important role in the inhibition of proteasome activity and the deterioration of synaptic connections within the brains of AD patients.

1.5 Ubiquitin Proteasome System and Tau Protein

Taking all of the evidence discussed so far, the ubiquitin proteasome system is an important candidate for being a therapeutic target for patients with AD. Specifically, it seems that the accumulation of tau protein within the cell may be one of the factors determining the progression of AD and other tauopathies as the accumulation of NFT is correlated with the level of dementia in AD patients (Arriagada et al, 1992; Hansson et al., 2006) and proteasome activity is found to be significantly decreased in patients with AD (Keller et al., 2000).

In the past, several groups have investigated the relationship between the proteasome degradation pathway and tau in cell culture and animal models. However, results from different studies seem to present conflicting results. Although most cell-free assays all seem to suggest that tau is directly degraded by the proteasome and that the presence of filamentous tau can inhibit the 20S proteasome in the brain (Keck et al., 2003), different in vitro and in vivo studies have reported inconsistent results regarding proteasome inhibition and tau turnover.

A study done by David and colleagues (2002) found that inhibition of the proteasome with lactacystin in SH-SY5Y neuroblastoma cells was able to inhibit the degradation of recombinant tau compared to control. Similarly, an in vivo study involving lactacystin injection into the hippocampi of rats to inhibit proteasome activity found an accumulation of total tau and both phosphorylated and non-phosphorylated forms of tau in the brain (Liu et al., 2008). Samples co-immunoprecipitated with tau for ubiquitin found an increased immunoreactivity for ubiquitin, suggesting the build up of tau within the rat hippocampi due to proteasome inhibition is highly ubiquitinated and is targeted for proteasome degradation. There was also an accumulation of RIPA-insoluble tau in lactacystin-treated brain samples, which the authors attributed to a decrease in tau solubility from proteasome inhibition, resulting in tau aggregation. These studies
support the notion that proteasome inhibition is involved in the accumulation of tau protein within the cell, promoting its aggregation and formation of NFT within the cell.

Conversely, Feuillette et al. (2005) found that inhibition of proteasome activity in SH-SY5Y cells with lactacystin, epoxomicin, and MG132 resulted in a significant decrease in endogenous tau expression, although there was an increase in ubiquitin immunoreactivity which confirmed that the ubiquitin-proteasome system was in fact inhibited. To investigate this in vivo, they used a mutant Drosophila line with impaired proteasome activity and demonstrated a decrease in total tau expression in those flies. Delobel and colleagues (2005) found similar results while also making use of SH-SY5Y cells that overexpress tau. They found that inhibition of the proteasome with MG132 and PSI was able to promote tau proteolysis rather than accumulation. Both groups suggest that although proteasome inhibition increased tau degradation within SH-SY5Y cells, tau may not be degraded directly by the proteasome; rather, inhibition of the proteasome increases the accumulation of other proteases involved in tau degradation, such as calpain.

1.6 Calpain and Tau Protein

Calpain is a calcium-dependent, non-lysosomal protease that is found ubiquitously in many cell types. It was originally speculated that potential calpain cleavage sites follow a P2-P1 rule. The P2-P1 rule states there is a preference of calpain to cleave target proteins possessing a Leu or Val at position P2 and Arg or Lys at P1 prior to the scissile bond (Hirao and Takahashi, 1984). However, more recent evidence seems to suggest that calpains do not actually recognize specific amino acid sequences for cleavage sites and actually recognize target proteins by its tertiary conformation (Cuerrier et al, 2005). Gail Johnson and colleagues have done a great deal of research looking at the relationship between calpain and tau degradation. They were the first to demonstrate that tau isolated from bovine brain can be degraded by calpain (Johnson et al., 1989) and calpain inhibitors were able to prevent this (Xie & Johnson, 1998). Results from other research groups support an interaction between calpain and tau (Park et al., 2007; Liu et al., 2011; Ferriera & Bigio, 2011). Phosphorylation of tau by protein kinase A was found to inhibit
the degradation of tau by calpain (Literalsky & Johnson, 1992), which is in accord with the accumulation of hyperphosphorylated tau in brains of AD patients.

In line with a role of calpain in tau degradation, Park and Ferreira (2005) found that incubation of pre-aggregated Aβ poteniated calpain activity, which increased the production of an N-terminal 17kDa tau fragment. Overexpression of this tau fragment in CHO (Chinese hamster ovary) cells and cultured hippocampal neurons was neurotoxic and induced apoptosis. Transgenic AD mice that show signs of neurodegeneration have also been shown to express this 17 kDa tau fragment (Roberson et al., 2007). This could be a possible pathway by which Aβ, in concert with tau, induces AD-related neurodegeneration.

Contrary to the study done by Park and Ferreira (2005), a recent follow up study to investigate this 17 kDa toxic fragment (Garg et al., 2010) suggests that the increased production of the 17 kDa tau fragment due to potentiation of calpain by Aβ is in fact a general response to calpain induction. Treatment with glutamate, a neurotransmitter that has been implicated in AD neurodegeneration, and a calcium-dependent calpain activator were able to generate the same tau fragment in neuronal cells, thereby abolishing a specific relationship between Aβ and this 17 kDa tau fragment in inducing neurodegeneration. Moreover, the specific 17 kDa tau fragment was also found to be non-toxic in CHO, N2a neuroblastoma cells, or cultured hippocampal neurons. Lastly, they were able to detect the presence of the 17 kDa tau fragment in the hippocampus of select AD patients, but also present in control patients. Although the evidence supports a role of calpain in mediating tau turnover, the specific role of calpain in inducing tau pathology in AD patients remains unclear.

It is important to note that a study done by Brown and colleagues (2005) contests this relationship between calpain and tau turnover. They showed that incubation of SH-SY5Y or primary rat hippocampal neurons with calpain inhibitor for up to 48hrs did not alter tau expression. The specific cleavage site or consensus sequence of calpain in tau has also never been reported (reviewed by Garg et al., 2010), making it difficult to determine if truncated or toxic isoforms of tau found in AD brain are a result of calpain cleavage. Also, many studies done by Johnson’s group (Johnson et al., 1989; Littersky & Johnson, 1992; Xie & Johnson, 1998) were conducted in cell-free assays using tau isolated from bovine brain, which do not take into
account the intricate interactions that are better addressed in cell and animal models.

Although a study conducted by Xie & Johnson (1998) did examine the effects of calpain on tau in PC12 rat adrenal medulla cells, they established a causative role for calpain in tau degradation by observing that calpain inhibitor CBZ-LLY-DMK blocked the effects of calpain induction by maitotoxin, a drug that increases calcium concentrations within the cell. A wide range of enzymes and proteases are calcium dependent, so induction of calpain activity by increasing calcium concentrations within the cell may also induce the activity of other enzymes and proteases involved in tau turnover; moreover, the effects of the calpain inhibitor may not be specific to calpain only (Kisselev et al, 2001; Myung et al., 2001).

Therefore, taking all the evidence presented thus far, there seems to be strong evidence in support of a link between calpain and tau turnover but whether calpain is a direct or indirect mediator of tau pathology in AD still requires further investigation.

1.7 Current Models for Investigating the Ubiquitin Proteasome System and Alzheimer’s Disease

A representative animal model for investigating the role of proteasome inhibition on the development and progression of AD must demonstrate the key aspects of AD pathology and chronic inhibition of the proteasome.

There are currently a large number of transgenic mouse models that display some, though not all, aspects of AD pathology. The most commonly used transgenic models target either the Mapt tau gene or App gene to promote tau or Aβ pathology, respectively. Although they succeed in presenting certain aspects of AD pathology in an in vivo animal model, they do not present all of the signs of AD as seen in human patients. Many groups have attempted to create transgenic mice that harbour both tau and Aβ pathology by crossing mutant tau and mutant APP transgenic mice with more enhanced pathology compared to single mutant animal models. TAPP mice (Lewis et al., 2001) revealed more prominent NFT pathology by 9 months of age compared to mice expressing mutant tau only while a study done by Ribe and colleagues (2005) found more
enhanced Aβ deposition in an age dependent manner and NFT formation in mice aged 25 months.

Another approach taken to create double mutant animal models was to inject several vector constructs into fertilized oocytes to create transgenic mice with a single homogenous transgene background (Oddo et al., 2003); in these mice, Aβ and tau pathology was evident in a progressive and age-dependent manner in the specific vulnerable brain regions seen in patients with AD. Although this is the best preclinical model researchers can utilize today, it still has its drawbacks. There were no signs of neuronal degeneration in this model and cognitive impairment was evident prior to the formation of NFT or senile plaques (Chin, 2011). This model is also unable to overcome the prevalent gender differences in the severity of neuropathology in Aβ-targeted mouse models of AD, with females exhibiting signs of AD pathology earlier than males (Carroll et al., 2007).

Another drawback of transgenic animal models was the presentation of motor deficits that complicate the use of behavioural tests. Fortunately, recent research advances have developed triple-transgenic mice that overcome the issue of motor deficits (Tatebayashi et al., 2002, Schindowski et al., 2006). For example, a mouse model developed by Schindowski and colleagues (2006) shows presence of hyperphosphorylated tau at common epitopes found in patients with AD, formation of NFT-like inclusions, and memory deficits at 10 months of age without signs of motor impairment.

The only animal model that genetically impairs proteasome activity was developed by John Mayer’s group (2008). Making use of a Cre-loxP recombination system to selectively inactivate Rpt2 of the proteasome, this model prevents proper opening of the α ring gate for translocation of targeted proteins toward the catalytic core, thereby impairing proper 26S proteasome activity.

There were signs of ubiquitin and α-synuclein inclusions although no aggregates of Aβ or tau were detectable. This line of transgenic animals also shows signs of growth retardation, impairment of normal neurological function, and they die after 3-4 months from birth from lack of appetite. Although this transgenic model may be suitable in the short term for investigating proteasome inhibition, it is not a representative model for AD. This model also does not specifically target the proteolytic activity of the proteasome and only inhibits the proteasome by
preventing access through the 20S catalytic core.

An in vitro study was done to genetically impair a proteolytic subunit of the proteasome by Li and colleagues (2004). By introducing a mutation at the Thr1Ala site of the β5 subunit, the chymotryptic activity of the proteasome was significantly impaired. Their group found this genetic mutation of the proteasome resulted in a build up of ubiquitin in HT4 mouse neuronal cells and hypersensitized them to oxidative stress induced by cadmium.

To our knowledge, there are currently no studies done looking at the effects of proteasome impairment and tau or Aβ pathology by genetic impairment of the proteasome. Much of the studies done on the relationship between the ubiquitin proteasome system and tau have made use of pharmacological inhibition of the proteasome, cell systems that overexpress tau or cell-free assays that incubate isolated recombinant tau with 20S proteasomes. A study done by David and colleagues (2002) looked at the effects of proteasome inhibition and both endogenous and recombinant tau but found a difference in the rate of degradation between the two types of tau, although they did not address this further. The inconsistent results between endogenous and recombinant tau reminds us to be especially vigilant with experiments done with overexpression of specific proteins. When investigating neurodegenerative diseases in cell culture or transgenic mice that overexpress the target of interest, it is important to be careful when interpreting the results as overexpression of any protein can also disrupt the normal protein homeostasis within the model system, resulting in a non-representative model for neurodegenerative disease. Overexpression may also promote protein aggregation into inclusions, as seen in Szeto and colleagues (2006) study where expression of green fluorescent protein tagged ubiquitin spontaneously formed aggregates in Hela cells. Taking these issues into consideration, our study looks to investigate the effects of proteasome inhibition on endogenous tau protein by developing a cell culture model to specifically target one of the three proteolytic activities of the proteasome and examine its effects on tau protein expression within the cell.
2  Research Aims and Hypothesis

The aim of this research thesis is to develop a representative model of chronic proteasome inhibition comparable to that seen in patients with Alzheimer’s disease (AD) and investigate the effects of impaired proteasomal activity on tau protein. Many current models make use of pharmacological agents to acutely and severely impair proteasome activity, which is not representative of the disease model of AD. Proteasome inhibition as seen in AD and other age-related neurodegenerative diseases is a gradual and chronic process with the severity of proteasome inhibition to increase with age. This is likely due to a decline in the cell’s ability to break down unwanted or damaged proteins and an accumulation of oxidative species within the brain and other tissues over time (Ding & Keller, 2006). Therefore, the effect of proteasome inhibition on AD progression is likely a mild, chronic effect, rather than an acute effect as seen with pharmacological incubation with proteasome inhibitors.

Also, a number of research groups make use of recombinant tau to overexpress tau isoforms that are considered more prone to aggregation or overexpress tau to magnifying the effects of their experimental treatments on changes in tau turnover. However, recombinant tau have been found to respond differently than endogenous tau under the same treatment conditions (David et al, 2002) and overexpression of any protein makes it more prone for aggregation within cell model systems, resulting in its spontaneous aggregation (Szeto et al., 2006).

Therefore, we aim to develop a cell culture model that expresses a genetic mutation of the β5 subunit of the proteasome to chronically impair its chymotryptic activity by 30-50%, comparable to what has been found in patients with AD (Keller et al., 2000). We will then investigate how the chronic inhibition of the β5 subunit contributes to changes in the overall proteolytic activity of the proteasome and how this affects the expression of endogenous tau protein within the cell. To compare the difference between chronic and acute inhibition of the proteasome, we will also treat our cells with pharmacological agents and examine whether acute inhibition with proteasome inhibitors will yield a different response. To the best of our knowledge, no other research group has looked into the effects of acute and chronic inhibition of the proteasome on endogenous tau turnover.
Oxidative stress has also been a major topic of interest for researchers due to accumulating evidence for the role of oxidative species in the development of neurodegenerative diseases, disruption of protein homeostasis and mitochondrial dysfunction (reviewed by Aksenova et al., 2005). Of particular interest to our group, it has been found that oxidative stress contributes to proteasome inhibition, especially relevant to age-related neurodegenerative diseases such as AD (Ding & Keller, 2006). Previous studies conducted in our laboratory found that although mild and chronic proteasome inhibition does not seem to affect normal cell turnover or function under basal conditions, impaired proteasome function alters the cell’s ability to recovery and respond to environmental stresses such as oxidative stress. By treatment of hydrogen peroxide, we will investigate whether chronic impairment of the proteasome and subsequent exposure to oxidative stress contribute to tau accumulation and its aggregation, comparable to aged individuals and patients of AD.

We hypothesize that the chronic impairment of proper proteasome degradation of protein within the cell will result in the accumulation of tau protein, contributing to its aggregation into inclusions. We also hypothesize that the accumulation of tau protein will be more prominent in cells exposed to oxidative stress, due to the cell’s inability to cope with the environmental stress.
3 Materials and Methods

3.1 Plasmids

The mutant β5 T1A vector construct was created by site-directed mutagenesis into the human wild-type (WT) β5 cDNA (a gift from Dr. Peter Kloetzel, Charite Hospital, Berlin). The WT and T1A vectors contain a carboxy-terminal c-myc and His6 epitope tag for detection of vector expression. The vectors were then inserted into the pWPI vector (Addgene plasmid 12254), modified to include a neomycin resistance gene; the pLVCT-tTR-KRAB vector (Addgene plasmid 11643); and pTRE2hyg vector (Clontech), which carries the hygromycin resistance gene.

3.2 Cell Culture

SH-SY5Y neuroblastoma cells (ATCC, Manassas, Virginia), HEK293 (ATCC), TR8-SH-SY5Y neuroblastoma cells (a gift from Dr. Takafumi Hasegawa, Tohoku University School of Medicine, Japan), GOTO neuroblastoma cells, and KNS epithelial cells (gifts from Dr. Paul Fraser, University of Toronto, Toronto) were maintained in Dulbecco’s modified eagle media (DMEM) containing 10% Tet-System Approved Fetal Bovine Serum (Cedarlane Laboratories, Burlington, Canada) or 10% Fetal Bovine Serum (Wisent, St. Bruno, Canada) (FBS) at 37°C in 5% CO2. Cells were maintained in 100 x 20mm tissue culture dishes (Sarstedt, Nümbrecht, Germany) and passaged using 1X trypsin/EDTA (Wisent) at 1 to 4 when they reached 80-90% confluency.

3.3 Transfection of β5 vector

Transfection of the pTRE2hyg vector into TR8-SH-SY5Y neuroblastoma cells were conducted using Lipofectamine™ 2000 (Invitrogen, Carlsbad, California) and Cell Line Nucleofector® Kit V (Lonza, Basel, Switzerland). For transfection using Lipofectamine™ 2000, 16ug of DNA was transfected into 3x10⁶ cells while transfection using the Nucleofector® Kit was conducted using the A-023 program to transfect 2.5ug of DNA into 4x10⁶ cells. Successfully transfected cells
were screened by growing in media containing 20μg/ml hygromycin B for 3 weeks.

3.4 Infection of β5 vector

The pLVCT-tTR-KRAB and pWPI lentivectors were infected into SH-SY5Y neuroblastoma cells by incubation with 3x10^6 cells at 37°C/5% CO₂ for 5 hours before exchanging to DMEM with 10% FBS. Successfully infected cells were screened by growing in media containing 150μg/ml G418 for 3 weeks.

3.5 Doxycycline Treatment

Expression of the WT and T1A mutant β5 in the pTRE2hyg and pLVCT-tTR-KRAB vectors was induced using 0.5-1μg/ml doxycycline (Sigma-Aldrich) for 5 days.

3.6 Differentiation of SH-SY5Y Neuroblastoma Cells

SH-SY5Y neuroblastoma cells expressing the wild-type and mutant β5- T1A vector were differentiated using DMEM with 1% FBS and 50ng/ml nerve growth factor (Alomone Labs, Jerusalem, Israel) (NGF) or 10μM retinoic acid (Sigma-Aldrich) (RA) for 6-10 days.

3.7 Pharmacological Inhibition of Proteasome Activity

Proteasome activity was inhibited by incubating differentiated cells in DMEM with 1% FBS and 20-50nM epoxomicin (Peptides International, Louisville, USA) or 0.5-3μM of MG132 (Peptides International) for 24hrs.

3.8 Induction of Mild Oxidative Stress in SH-SY5Y Neuroblastoma Cells

Oxidative stress was induced in differentiated SH-SY5Y cells by incubating cells in DMEM containing 1% FBS and 0.5-3mM H₂O₂ (Sigma-Aldrich) for 1hr, followed by a 24hr recovery period in differentiation media.
3.9 Proteasome Activity Assay

Cells were lysed in cell lysis buffer (20mM Tris, pH 7.2, 1mM EDTA, 1mM NaN₃, 1mM DTT, and protease inhibitor cocktail (Sigma-Aldrich) on ice and centrifuged for 5min at 20,000g. The supernatants were quantified in triplicate in black polystyrene 96-well plates (Corning, New York, USA). Background activity levels were controlled for by incubation of duplicate samples with 5uM MG132. Plates were incubated at 4°C for 1hr before addition of fluorogenic substrates N-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin (Suc-LLVY-AMC) (Enzo Life Sciences, Farmingdale, USA) for chymotryptic activity, butoxycarbonyl-Leu-Arg-Arg-amido-4-methylcoumarin (Boc-LRR-AMC) (Enzo Life Sciences) for tryptic activity, and benzoyloxycarbonyl-Leu-Leu-Glu-amido-4-methylcoumarin (Z-LLE-AMC) (Cedarlane) for caspase-like activity at 20uM. After addition of the fluorogenic substrates, the plates were incubated at 37°C for 30mins and then read on a fluorescence microplate reader (Perkin Elmer, Waltham, USA) at 390nM excitation and 460nM emission. The level of fluorescence measured subtracted from background is taken as the level of proteolytic activity at each of the three proteasome catalytic sites.

3.10 Glycerol Density Gradient Centrifugation

SH-SY5Y neuroblastoma cells expressing the wild-type and mutant β5- T1A vector were harvested in cell lysis buffer and centrifuged for 5min at 20,000g. The supernatant fraction was separated on a 5-40% linear glycerol gradient prepared in cell lysis buffer. Gradients were centrifuged for 15hrs at 35,000g. The gradient was divided into 11 fractions and 100ul of each fraction was precipitated using ethanol precipitation and resuspended in sample buffer for separation on SDS-PAGE.

3.11 Western Blotting

Cells were lysed in cell lysis buffer and protein concentration of the soluble fraction was determined using the BCA Assay (Thermo Fisher Scientific, Rockford,USA). The insoluble fraction was further lysed in 6M urea and 2% SDS buffer. The pellets were sonicated at 15% amplitude for 30s each using the sonic dismembrator model 500 (Thermo Fisher Scientific) to
fully suspend the pellet into buffer. Laemmli sample buffer (60mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.01% bromophenol blue) was added to 30ug of protein from the soluble fraction and 50ug from the insoluble fraction and heated at 95°C for 5mins before separation on SDS-PAGE using 10% polyacrylamide gels and transferred to nitrocellulose membranes (Pall, Port Washington, USA). Membranes were blocked using 5% evaporated milk (Carnation, Markham, Canada) in Tris Buffered Saline (150mM NaCl, 50mM Tris, pH 7.4) with 0.5% TWEEN 20 (Sigma-Aldrich) (TBST). Membranes were incubated in primary antibodies in 2.5% milk in TBST for 1hr at room temperature or overnight at 4°C. Three washes of TBST, 5min each, were performed before incubation with secondary antibody in 2.5% milk in TBST for 1hr at room temperature. Membranes were visualized using ECL-Plus (GE Healthcare, Baie d’Urfe, Canada) after three 5min washes with TBST. Quantification of the bands was done using the Storm 860 molecular imager (GE Healthcare) and Imagequant 5.1 software (GE Healthcare).

3.12 Antibodies

Primary antibodies used include anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody (monoclonal #6C5 from Meridian Life Science, Inc., Saco, USA), anti-tetra-HIS for detection of the His<sub>6</sub> epitope (monoclonal #34670 from Qiagen, Venlo, Netherlands), anti-phospho-akt (polyclonal #9271 from Cell Signaling Technology, Danvers, USA), anti-Tet-R (polyclonal #ab14075 from Abcam, Cambridge, England), anti-proteasome beta subunit 2 (monoclonal #ab22650 from Abcam)(psmb2), anti-tau (polyclonal #A0024 from DAKO, Glostrup, Denmark) and anti-ubiquitin (polyclonal #Z0458 from DAKO).
4 Results

4.1 Establishment of a Suitable Cell Culture Model for Genetic Inhibition of the Proteasome

To establish a more representative model for chronic proteasome inhibition in tauopathies, we generated a vector carrying the mutant threonine 1 to alanine (T1A) β5 subunit to chronically inhibit the chymotryptic activity of the proteasome. The loss of the threonine residue impairs the chymotryptic activity of the β5 subunit in two ways. Firstly, the threonine residue acts as the nucleophile in the catalytic attack of the β5 subunit in peptide cleavage and mutation of the threonine residue to alanine impairs the β5 catalytic activity. Secondly, the β subunits of the proteasome are arranged into the proteasome complex as propeptides. Autocatalysis of the propeptides at two different cleavage sites reveals the active site and promotes maturation of the catalytic β subunits. One of the cleavage sites of the β5 subunit is at Thr1 and the T1A mutation prevents the proper cleavage of the propeptide into the mature subunit, resulting in full incorporation of the functionally inactive β5 subunit that retains 8-9 additional amino acids compared to the wild-type protein (Li et al., 2005). The expression of the T1A mutant impairs the chymotryptic activity of the proteasome by ~40%, which is similar (30-50% inhibition of chymotryptic activity) to that reported for postmortem Alzheimer’s disease brains (Keller et al., 2000).

4.1.1 Determining a Suitable Cell Type for Establishing a Cell Culture Model for Genetic Inhibition of the Proteasome

Since we were interested in assessing the effects of proteasome dysfunction on tau levels, our initial experiments focused on identifying a cell line with a measurable quantity of endogenous tau. Therefore, we tested three different cell types for the generation of our cell culture model: SH-SY5Y neuroblastoma cells, GOTO neuroblastoma cells, and KNS epithelial cells. SH-SY5Y neuroblastoma cells expressed the highest levels of endogenous tau compared to GOTO neuroblastoma cells and KNS epithelial cells (Figure 4.1) and so we decided to use SH-SY5Y neuroblastoma cells as our model for proteasome deficiency.
Figure 4.1: Expression of endogenous tau in GOTO neuroblastoma, KNS epithelial, and SH-SY5Y neuroblastoma cells.

Untreated GOTO neuroblastoma, KNS epithelial, and SH-SY5Y neuroblastoma cells were harvested under standard protocol. 30ug of protein was run on western blot and probed for tau (1:100000) and GAPDH (1:5000) for loading control. SH-SY5Y cells had the highest expression of tau compared to GOTO and KNS cells, making them the more suitable cell line for our experiments.
4.1.2 Determining the Proper Expression System for Establishing a Cell Culture Model for Genetic Inhibition of the Proteasome

Expression using an inducible system would allow for tighter and more specific temporal regulation of our gene of interest. The level of vector expression can also be manipulated with an inducible system. Moreover, the Li et al. (2004) study using stably transfected SH-SY5Y cells reported that expression of the T1A β5 mutant was 50-fold lower than the WT β5 protein. We speculated that this low level of mutant expression was due to its inherent toxicity rather than stability, such that cells with the lowest expression level would be more likely to survive selection. Therefore, inducible expression allows cells to be selected passaged with minimal T1A β5 expression, and induced only as experimentally desired. We investigated several systems for expressing our mutant T1A β5 subunit in SH-SY5Y cells: a two vector inducible transfection expression system (TR/pTREhyg), a single vector inducible lentiviral expression system (pLVCT-tTR-KRAB), and a single vector lentiviral stable expression system (pWPI) (Figure 4.2).

The two vector transfection inducible system involves the expression of the pcDNA6/TR© regulatory plasmid, encoding for the Tet repressor (TetR), and the expression of an inducible plasmid containing the mutant β5 T1A gene, the pTRE2hyg plasmid. Under basal conditions, the TetR binds to the Tet operator, preventing the expression of our gene of interest. Upon incubation with doxycycline, the TetR is released from the Tet operator, allowing for the transcription of the target gene. SH-SY5Y neuroblastoma cells expressing the TetR protein (TR8-SH-SY5Y) were generously donated to us by Dr. Hasegawa from the Tohoku University of Medicine. We then transfected the empty pTRE2hyg vector (M) as control, the pTRE2hyg vector containing the wild-type β5 (WT) and the pTRE2hyg vector containing the mutant T1A β5 (T1A) into TR8-SH-SY5Y cells.

Although there was clear expression of the TetR protein within our cells, we were not able to detect expression of wild-type (WT) or mutant T1A β5 by probing for the His<sub>6</sub> affinity tag (Figure 4.3).

We then attempted to express our WT and mutant T1A β5 in the single vector lentiviral inducible
system, pLVCT-tTR-KRAB. In the absence of doxycycline, the tTR-KRAB fusion protein binds to the tet operator expressed within the pLVCT sequence but upon induction by doxycycline, the tTR-KRAB falls off the tet operator, allowing for transcription of the gene of interest, which in our case would be either the WT or T1A β5 proteasome subunit. Using lentiviral infection, we infected an empty pLVCT-tTR-KRAB vector containing the GFP gene (M), a vector containing the WT β5 and one containing the mutant T1A β5 vector into both HEK293 cells and SH-SY5Y cells. Although we could not select for successful clones by use of antibiotics with this vector, we could visualize the infection rate of the lentivector by examination of GFP expression in the cells infected with the vector carrying the GFP gene. After induction with doxycycline, we found a very low infection rate of the pLVCT-tTR-KRAB vector into both HEK293 and SH-SY5Y cells (Figure 4.4A) as seen by the low number of cells expressing GFP under fluorescence imaging and no expression of the His₆ affinity tag by SDS-PAGE analysis (Figure 4.4B).

Lastly, we expressed our WT and mutant T1A β5 vector in the pWPI stable expression lentiviral system. This expression system contains a neomycin resistance gene for selection of successfully infected cells with the G418 antibiotic. After infection of the pWPI plasmid into SH-SY5Y cells, we were able to detect expression of both the WT and T1A β5 subunit by expression of the His₆ affinity tag by SDS-PAGE (Figure 4.5) within 48hrs.

Since the mutant T1A β5 subunit is not autcatalytically cleaved at the second cleavage site on the propeptide, it retains an addition 8-10 residues upstream of the Thr1, resulting in a slightly larger T1A β5 subunit than wildtype. Therefore, the β5T1A subunit has slightly lower mobility when visualized by gel electrophoresis and western blotting (Figure 4.5).

To ensure that our WT and T1A β5 subunits are not only expressed in SH-SY5Y cells, but also incorporated into proteasome complexes, we examined whether our His₆ epitope tagged β5 subunits can be detected in proteasome complexes by separating large protein complexes by molecular weight on a linear glycerol density gradient. If the WT and T1A β5 subunits are successfully incorporated into proteasome complexes, they should be detected within the high molecular weight fractions corresponding to about 350kDa for the half proteasome complex and 700kDa for a fully assembled proteasome (Gerards et al., 1998).

As seen in Figure 4.6, mature WT and T1A β5 subunits were found in high molecular weight
fractions corresponding with assembled proteasome complexes and they also co-eluted with endogenous β2 subunit on a glycerol density gradient, indicating successful incorporation of our WT and T1A β5 subunits into proteasome complexes within the cell. The maturation of the full length β5 propeptide into mature β5 subunits also indicates the incorporation of our β5 subunits into the proteasome as cleavage and maturation of the propeptide does not occur until the propeptide has been fully incorporated into proteasome complexes (Schmidtke et al., 1996).

Next, we wanted to test whether the successful expression and incorporation of mutant T1A β5 subunit resulted in impaired chymotryptic activity within the SH-SY5Y cells.

There was a 40% decrease in chymotryptic activity in the mutant T1A cells compared to both control (M) and WT cells, with no statistically significant changes in either tryptic or caspase-like activity (Figure 4.7). Therefore, the inhibition of the proteasome by the T1A mutation was specific to the chymotryptic activity of the proteasome and induced a 40% decrease in β5 activity that is comparable to the decrease measured in Alzheimer’s disease brains (Keller et al., 2000).

Therefore, we conclude that infection of the WT and T1A β5 vector into SH-SY5Y neuroblastoma cells using the stable expression pWPI lentiviral system results in successful expression of the β5 genes, incorporation of the WT and T1A β5 subunits into proteasome complexes, and significant impairment of the β5 chymotryptic activity of the proteasome in the mutant T1A cell line.
Figure 4.2: Schematic representation of the vector plasmids used for expression of the wildtype and mutant T1A β5 subunit.
Figure 4.3: No expression of the pTRE2hyg vector in TR8-SH-SY5Y neuroblastoma cells after induction with doxycycline for 5 days.

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Transfection of the pTRE2hyg vector to express the empty vector (M), WT β5 (WT) and T1A β5 mutant (T1A) in TR8-SH-SY5Y neuroblastoma cells was done using Lipofectamine 2000 and Cell Line Nucleofector Kit V. Successful clones were selected for by incubating the cells in growth media containing 20ug/ml hygromycin B for 3 weeks. Cells were incubated in 0.5ug/ml and 1ug/ml of doxycycline for 5 days to induce expression of the vector then harvested using standard procedures for analysis on western blot. 30ug of protein was loaded on gels and probed with antibodies recognizing the His$_6$ epitope tag (tetra-his) (1:1000), tetR protein (1:1000) and GAPDH (1:5000). There were no detectable amounts of the pTRE2hyg vector, as determined by the expression of the His$_6$ affinity tag.
SH-SY5Y neuroblastoma and HEK293 cells infected with the pLVCT-tTR-KRAB inducible lentivector were incubated in media containing 1μg/ml doxycycline for 5 days to induce expression of the M, WT and T1A β5 mutant vector. (A) Expression of the control (M) lentivector was visualized using fluorescence imaging of GFP. (B) Expression of the WT and T1A β5 mutant vector in SH-SY5Y neuroblastoma cells were detected by harvesting control (Θ) and induced cells (Tet-On) using standard protocol and running 30μg of lysate on western blot. Western blots were probed for the His$_6$ epitope tag (1:1000) and GAPDH (1:5000) for loading control. There was a very low level of GFP expression in both SH-SY5Y and HEK293 cells, indicative of successful induction but low infection rate of the pLVCT-tTR-KRAB lentivector in both cell types. There was no detectable expression of the His$_6$ affinity tag by probing with the anti-tetra-his antibody on western blot, which suggest a low infection rate of the WT and T1A β5 mutant vector in SH-SY5Y neuroblastoma cells.
Figure 4.5: Expression of the wildtype and mutant T1A β5 subunit in SH-SY5Y neuroblastoma cells infected with the pWPI lentivector.

SH-SY5Y neuroblastoma cells infected with the stable expression pWPI vector was harvested by standard protocol and 30μg of lysate was run on western blot. Western blots were probed for expression of the His$_6$ affinity tag with the tetra-his antibody (1:1000) and GAPDH (1:5000) for loading control. There were detectable levels of the His$_6$ affinity tag in cells expressing both the WT and T1A β5 vector, with the expression of the WT vector triple that of the T1A vector.
Figure 4.6: Incorporation and full maturation of WT and T1A β5 mutant subunits in SH-SY5Y cells infected with the pWPI stable expression lentivector.

SH-SY5Y neuroblastoma cells infected with the pWPI lentiviral vector expressing WT and T1A β5 mutant subunits, with a mock (M) vector as control. The cells were harvested using standard protocol and lysates were separated on a linear glycerol density gradient (40%-5% v/v) into 11 fractions. Equal amount of proteins from each fraction were concentrated by ethanol precipitation, separated by SDS-PAGE and transferred to nitrocellulose membrane for western blotting. Membranes were probed for endogenous β2 subunits (psmβ2) (1:1000) and expression of the WT and T1A β5 mutant subunits using the tetra-his antibody (1:1000). Expression of the WT and T1A β5 mutant subunits were detected mostly as mature forms, which run at 28 and 32 kDa, respectively.
Figure 4.7: Significant impairment of chymotryptic activity in SH-SY5Y cells expressing the mock control (M), wildtype (WT), and mutant T1A β5 subunit of the proteasome.

SH-SY5Y neuroblastoma cells expressing the mock, WT or T1A β5 mutant proteasome subunit were assayed for chymotryptic, tryptic and caspase-like proteasome activity. Proteasome activity in each cell type was normalized to the activity in mock-infected cells. Cells infected with the T1A β5 vector showed significant inhibition of chymotryptic activity, with no significant changes in tryptic or caspase-like activity. Values are means ± SEM. ** denotes significance of p<0.01 between mocked untreated cells to each experimental condition by one way ANOVA test (n=3).
4.2 Differentiation of SH-SY5Y Neuroblastoma Cells Increases Tau Expression and Promotes a More Neuronal Morphology

Differentiation of SH-SY5Y cells has been found to promote a more mature neuronal phenotype biochemically and morphologically (Hong-Rong et al., 2010), making them a more neuronal-like model for research purposes. Therefore, we differentiated the SH-SY5Y cells with media containing reduced serum, nerve growth factor (NGF) and retinoic acid (RA) and measured the expression of endogenous tau, a protein commonly used as a neuronal marker and our protein of interest.

To ensure our cells were differentiated after treatment with reduced serum and RA, we probed the cell lysates for expression of phospho-akt, a common marker of differentiation. We found expression of phospho-akt in cells exposed to RA and not in control. Therefore, differentiation of SH-SY5Y neuroblastoma cells was achieved by exposure to reduced serum and 10uM RA for 7 days (Figure 4.9A).

After differentiation of SH-SY5Y cells with reduced serum and NGF or RA, endogenous tau levels were increased by over 50%. Since tau is mostly found in neurons and less so in other cell types, the increase in tau levels indicates a more neuronal biochemical phenotype of SH-SY5Y cells following differentiation (Figure 4.9B).

To investigate whether differentiation also promotes a more neuronal morphology in SH-SY5Y cells, we differentiated our SH-SY5Y cells infected with our empty pWPI vector (M), wild-type β5 (WT), and T1A β5 mutant (T1A). In addition to a comparable increase in tau levels in uninfected cells (Figure 4.9B), we also saw a more neuronal morphology as indicated by the extensive neurite growth arborization (Figure 4.9C).

Based on the increased level of endogenous tau and more neuronal morphology, we conclude that differentiation of our pWPI infected SH-SY5Y neuroblastoma cells in DMEM with 1% FBS and 10uM RA for 7 days provided us with a suitable cell model for investigating the effects of proteasome inhibition on tau protein.
Figure 4.8: Increased level of endogenous tau in SH-SY5Y cells after differentiation with nerve growth factor (NGF) and retinoic acid (RA).

SH-SY5Y neuroblastoma cells were differentiated in Dulbecco’s modified eagle media (DMEM) with 1% Fetal Bovine Serum (FBS) and 50ng/ml NGF or 10uM RA for 6 to 10 days. The cells were then lysed and 30ug of protein was separated in SDS-PAGE. Western blots were probed for tau (1:100000) and for GAPDH (1:5000) as a loading control. Undifferentiated cells were used as baseline and the relative ratio of tau levels was plotted against length of differentiation treatment. Tau expression was nearly two-fold in differentiated cells.
Figure 4.9: (A) Differentiation of SH-SY5Y neuroblastoma cells with reduced serum and retinoic acid. (B) Increased level of endogenous tau in differentiated SH-SY5Y cells infected with the empty pWPI lentivector (M), and pWPI vector carrying the wild-type (WT) and mutant T1A β5 (T1A). (C) Differentiation with reduced serum and retinoic acid promoted the development of extensive processes in infected SH-SY5Y neuroblastoma cells.
SH-SY5Y neuroblastoma cells infected with the pWPI mock, WT and T1A vector was differentiated in DMEM containing 1% FBS and 10μM RA for 7 days. The morphology of the cells was observed using brightfield imaging. Thirty microgram of protein was then separated on SDS-PAGE and probed for phospho-akt (1:1000), tau (1:100000) and GAPDH (1:5000) by western blotting. Differentiation with reduced serum and RA resulted in a higher level of tau and promoted the elongation of neurites in infected SH-SY5Y cells.
4.3 Pharmacological Inhibition of the Proteasome to Enhance the Effects of Proteasome Inhibition by T1A on Tau Protein

Previous results from our laboratory have found that chronic impairment of the chymotryptic activity in HEK293 cells expressing B5 T1A did not affect cell viability or show any clear signs of pathology compared to control (D’Souza & Tandon, unpublished data). However, when exposed to additional oxidative stressors such as H$_2$O$_2$ or FeSO$_4$, cells with impaired chymotryptic activity showed a reduced ability to recovery compared to mock and wild-type cells. After exposure to oxidative stress, cells were given a 24hr recovery period to recover from the H$_2$O$_2$ or FeSO$_4$ insult. It was found that T1A mutant cells had a significantly higher accumulation of ubiquitinated proteins and oxidized proteins as well as reduced cell viability compared to control (D’Souza & Tandon, unpublished data).

Therefore, we subjected our cells to three additional pharmacological treatments to bring out any subtle changes in tau protein in differentiated SH-SY5Y cells. We tested the effects of two proteasome inhibitors, epoxomicin and MG132, to examine the whether T1A cells were sensitized to effects of severe and acute proteasome inhibition and whether this might exacerbate any effects on tau protein. We used both epoxomicin and MG132 as epoxomicin is an irreversible inhibitor of proteasomal chymotryptic activity with no other known targets, while MG132 is a reversible inhibitor that broadly acts at all three proteolytic sites of the proteasome, although with a preference for inhibiting β5 activity (Kisselev & Goldberg, 2001). Since oxidative stress has been found to inhibit proteasome activity and may also contribute to AD, we also exposed the cells to oxidative stress with H$_2$O$_2$ treatment to examine for any changes in endogenous tau within the cell (reviewed by Keller et al., 2000; Grimm et al., 2010).

4.3.1 Significant Accumulation of Ubiquitinated Proteins with Epoxomicin and MG132 Treatment

To determine a suitable concentration of epoxomicin and MG132 for differentiated SH-SY5Y cells, we treated the cells with various concentrations of the two proteasome inhibitors for 24hrs
and then examined changes in tau and ubiquitinated proteins by SDS-PAGE.

Although there were no significant changes in tau under any of the treatment conditions, there was a significant accumulation of ubiquitinated proteins in cells treated with 20 (p<0.01), 30 and 50nM (p<0.001) of epoxomicin and 0.5 (p<0.001) and 1uM (p<0.01) of MG132 for 24hrs. This indicates that pharmacological inhibition of the 26S proteasome by epoxomicin and MG132 significantly inhibited proteasomal function, resulting in an accumulation of ubiquitinated proteins targeted for proteasome degradation. The accumulation of ubiquitinated proteins reached maximal between 20 to 30nM of epoxomicin and at 0.5uM of MG132. Therefore, we used 25nM of epoxomicin and 0.5uM of MG132 in the following experiments.
Figure 4.10: Significant increase in ubiquitinated protein expression but not in tau expression in differentiated SH-SY5Y cells treated with various concentrations of epoxomicin and MG132.

SH-SY5Y neuroblastoma cells were first differentiated with reduced serum and retinoic acid for 7 days and then treated with various concentrations of epoxomicin and MG132. The cells were then harvested and 30μg of protein was loaded on gels. (A) Western blots were probed for ubiquitin (1:4000), tau (1:100000) and GAPDH (1:5000) for loading control. Control levels of ubiquitin and tau expression were quantified by densitometric analysis of differentiated cells with no additional treatment. The ratio of protein expression was plotted against treatment concentration of epoxomicin (B) and MG132 (C). There was a significant increase in ubiquitin immunoreactivity in cells treated with epoxomicin and MG132 (p<0.01 and 0.001 as indicated) but no significant changes in tau. Values are means ± SEM. ** denotes p<0.01 and *** denotes p<0.001 between mocked untreated cells to each experimental condition by two way ANOVA test and Bonferroni multiple comparison post-test (n=3).
4.3.2. Significant Accumulation of Tau in Differentiated SH-SY5Y Cells Treated with H$_2$O$_2$

To investigate whether exposure to oxidative stress by H$_2$O$_2$ would yield a similar response, we treated differentiated SH-SY5Y cells with various concentrations of H$_2$O$_2$ and found a significant increase in tau expression (p<0.05) in cells treated with 0.5 and 1mM H$_2$O$_2$ but no changes in ubiquitin immunoreactivity (Figure 4.11).

Although the accumulation of endogenous tau within the cells cannot be attributed to direct inhibition of proteasome activity, it is clear that oxidative stress in SH-SY5Y cells did not affect ubiquitin levels within the cell, suggesting the increase in tau from oxidative stress acts through an ubiquitin-independent pathway at our treatment concentrations. The effects of H$_2$O$_2$ on tau accumulation seem to plateau at 0.5 mM, and so we used 0.5 mM H$_2$O$_2$ for all subsequent experiments.
Figure 4.11: Increased expression of tau in differentiated SH-SY5Y cells exposed to H$_2$O$_2$, with no significant changes in ubiquitin accumulation.

SH-SY5Y neuroblastoma cells were differentiated in media containing 1% FBS and 10uM RA for 7 days prior to treatment with different concentrations of H$_2$O$_2$ for 1 hr, followed by a 24hr recovery period in differentiation media. The cells were then harvested using standard protocol and 30ug of protein was run on western blot. Western blots were probed for tau, ubiquitin, and GAPDH for loading control. Relative levels of tau and ubiquitin was plotted against H$_2$O$_2$ treatment concentration, with differentiated cells subjected to no H$_2$O$_2$ treatment as baseline. There was a significant increase in tau levels in cells treated with 0.5 and 1mM H$_2$O$_2$ (p<0.05), and no significant changes in ubiquitin accumulation under all treatment concentrations. Values
are means ± SEM. * denotes p<0.05 between mocked untreated cells to each experimental condition by two-way ANOVA test and Bonferroni multiple comparison post-test (n=3).
4.4 Significant Inhibition of Chymotryptic Activity by Genetic and Pharmacological Approaches Did Not Affect Soluble Tau Expression

To investigate the effects of genetic and pharmacological inhibition of the proteasome on tau protein, we examined the relationship between the three proteolytic activities of the proteasome on tau levels and ubiquitin accumulation. We first established a genetic T1A mutation of the β5 subunit of the proteasome, to specifically inhibit the chymotryptic activity of the proteasome, through lentiviral infection with the pWPI stable expression system. We established three cell lines stably expressing either mock empty pWPI vector as control (M), wild-type β5 subunit (WT), or the T1A mutant (T1A). We then differentiated the cells to promote tau expression and induce a more neuronal phenotype in SH-SY5Y cells. Lastly, we treated the cells with three pharmacological agents, epoxomicin, MG132, and H₂O₂, to simulate the effects of acute and severe proteasome inhibition and oxidative stress on tau protein expression. Pharmacological treatment with proteasome inhibitors also tested for any subtle changes in SH-SY5Y neuroblastoma cells from chronic inhibition of β5 activity of the proteasome.

4.4.1 Significant Inhibition of Chymotryptic Activity in Differentiated SH-SY5Y Cells Under All Treatment Conditions

There was a significant inhibition of chymotryptic activity by ~40% in the mutant T1A cell line (p<0.001) compared to both mock and wild-type (Figure 4.12), indicating the successful inhibition of the proteasome by the genetic mutation of the β5 subunit. There was also a significant decrease in chymotryptic activity in all cell lines under all treatment conditions compared to untreated mock cells (p<0.001) (Figure 4.12). Chymotryptic activity decreased to about 10% of untreated mock cells in cells treated with epoxomicin and MG132, and to 60% with exposure to H₂O₂ compared to cells without any treatment. Inhibition of the chymotryptic activity by ~40%, as seen in our mutant T1A cell line and cells exposed to oxidative stress, is comparable to what Keller and colleagues (2000) found in postmorten AD brains.
Figure 4.12: Significant inhibition of chymotryptic activity in T1A β5 mutant expressing cells and SH-SY5Y neuroblastoma cells treated with epoxomicin, MG132 and H$_2$O$_2$.

SH-SY5Y neuroblastoma cells infected with the mock, WT and T1A β5 mutant vector were differentiated in reduced serum media and 10uM RA for 7 days then subsequently treated with 25nM epoxomicin for 24hrs, 0.5uM MG132 for 24hrs, or 0.5mM H$_2$O$_2$ for 1hr with a 24hr recovery period in differentiation media. The cells were harvested using standard protocol and lysates were assessed using the proteasome activity assay. Activity in differentiated mock-infected cells with no additional treatment was used as controls and relative chymotryptic activity was plotted against transgene and treatment condition. There was a significant inhibition of chymotryptic activity from genetic mutation of the β5 subunit and from pharmacological inhibition by epoxomicin, MG132 and H$_2$O$_2$. Values are means ± SEM. *** denotes p<0.001 between mocked untreated cells to each experimental condition by two way ANOVA test and Bonferroni multiple comparison post-test (n=5).
4.4.2 Significant Increase In Tryptic Activity in Mutant T1A Cells Treated with MG132

To test whether genetic or pharmacological inhibition of the proteasome resulted to changes in other proteolytic activities, and to what extent, we also tested for changes in tryptic activity in T1A mutant cells and in cells exposed to epoxomicin, MG132 and H₂O₂.

There appeared to be a trend to increased tryptic activity in mutant T1A cells compared to M (Figure 4.13), although the difference did not reach statistical significance, and could reflect a compensatory response to the chronic inhibition of chymotryptic activity of the β5 subunit. The large variation between each treatment may indicate that our proteasome assay for tryptic activity may not be accounting for changes in tryptic activity of the proteasome alone. Although the use of fluorogenic substrates to measure proteasome activity is still one of the most common and accepted techniques used today, the substrate used to measure tryptic activity, Boc-LRR-AMC, has a low signal-to-noise ratio compared to substrates used for measuring chymotryptic activity (O’Brien et al., 2006). Although we tried to control for background proteasome activity within the assay, Boc-LRR-AMC may still be cleaved by proteasome-independent proteases within the cell that we were not able to control for, resulting in a large variation on our results.
Infected SH-SY5Y neuroblastoma cells were differentiated in media containing 1% FBS and 10uM RA for 7 days. The cells were then treated with 25nM epoxomicin for 24hrs, 0.5uM MG132 for 24hrs, or 0.5mM H$_2$O$_2$ for 1hr with a 24hr recovery period in differentiation media. The cells were harvested using standard protocol and lysates were assessed using the proteasome activity assay. Activity in differentiated mock cells with no additional treatment was used as controls and relative tryptic activity was plotted against cell type and treatment condition. There were no significant changes in tryptic activity between M, WT and T1A cells under each treatment condition. Tryptic activity in mutant T1A cells treated with MG132 was significantly higher than untreated M cells (p<0.05), but not significantly different to untreated T1A cells. Values are means ± SEM. * denotes p<0.05 between mocked untreated cells to each experimental condition by two way ANOVA test and Bonferroni multiple comparison post-test (n=7).
4.4.3 Significant Inhibition of Caspase-like Activity of the Proteasome with Epoxomicin and MG132 Treatment

To investigate whether a similar trend in increased caspase-like activity was found in T1A cells as we found with tryptic activity, we tested for changes in caspase-like activity in pWPI infected cells with and without additional treatment of proteasome inhibitors.

There was a significant decrease in caspase-like activity of the proteasome with epoxomicin and MG132 treatment (p<0.001), with no significant differences amongst the three cell lines (Figure 4.14). Genetic inhibition of the β5 subunit does not seem to have any detectable effects on caspase-like activity of the proteasome, with all three cell lines displaying similar levels of caspase-like activity. Chymotryptic and caspase-like activity were most susceptible to the effects of epoxomicin and MG132 treatment, while H₂O₂ treatment seemed to only affect the chymotryptic activity of the proteasome.
Figure 4.14: Caspase-like activity of differentiated pWPI lentiviral infected SH-SY5Y neuroblastoma cells treated with epoxomicin, MG132 and H₂O₂.

SH-SY5Y neuroblastoma cells infected with the mock (M), WT, and T1A β5 mutant lentivector were differentiated with reduced serum media containing 10μM RA for 7 days. The cells were subsequently exposed to 25nM epoxomicin for 24hrs, 0.5μM MG132 for 24hrs, or 0.5mM H₂O₂ for 1hr with a 24hr recovery period in differentiation media. Cells were harvested and proteasome activity was assessed by running the cell lysates on a proteasome activity assay. Activity in differentiated mock cells with no additional treatment was used as control and relative caspase-like activity was plotted against cell type and treatment condition. There was a significant decrease in caspase-like activity in cells treated with epoxomicin or MG132 (p<0.001). Values are means ± SEM. *** denotes p<0.001 between mocked untreated cells to each experimental condition by two way ANOVA test and Bonferroni multiple comparison post-test (n=5).
4.5 Accumulation of Ubiquitinated Proteins in Cells Treated with Epoxomicin and MG132

After establishing the proteasome activity profiles of pWPI infected SH-SY5Y cells under the various treatment conditions, we tested for the effects of genetic and pharmacological inhibition of the proteasome on changes in ubiquitin and tau levels. This will allow us to compare how changes in the three different proteolytic activities of the proteasome may affect the cellular levels of tau and ubiquitin.

We found a significant accumulation of ubiquitinated proteins in cells treated with epoxomicin and MG132 (Figure 4.15A&B), but not in cells exposed to oxidative stress. This supports the notion that oxidized proteins may not be degraded through the 26S proteasome, but may in fact go through an ATP-ubiquitin independent pathway. There were also no significant differences between the mock, wildtype or mutant T1A cell line, indicating chronic impairment of β5 activity by 40% (Figure 4.12) is not sufficient to promote accumulation of ubiquitin conjugates.
Figure 4.15: Accumulation of ubiquitinated proteins in response to epoxomicin and MG132 treatment for 24hr.
SH-SY5Y neuroblastoma cells infected with the mock (M), WT, and T1A β5 mutant lentivector were differentiated with media containing 1% FBS and 10uM RA for 7 days, after which the cells were exposed to 25 nM epoxomicin for 24 hrs, 0.5 uM MG132 for 24 hrs, or 0.5 mM H$_2$O$_2$ for 1 hr with a 24 hr recovery period in differentiation media. Cells were harvested using standard protocol and 30 ug of protein was run on western blot. NP-40 insoluble pellets were subsequently lysed in 2% SDS/6M urea buffer before running on western blot. (A) Western blots were probed for ubiquitin (1:4000), tau (1:50000 for insoluble and 1:100000 for soluble tau), proteasome beta subunit 2 (PSMβ2) (1:1000), His$_6$ affinity tag with the tetra-his antibody (1:1000), and GAPDH (1:5000) for loading control. Densitometric analysis of differentiated mock-infected cells exposed to no additional treatment was used as control and relative protein levels was plotted against cell type and treatment condition. Values are means ± SEM. *** denotes p<0.001 between mocked untreated cells to each experimental condition by two way ANOVA test and Bonferroni multiple comparison post-test (n=7).
4.6 Accumulation of Soluble Tau in Cells Treated with MG132 in a Proteasome Independent Manner

If tau protein was preferentially degraded by the chymotryptic activity of the proteasome, we would expect a significant accumulation of tau protein in cells treated with both epoxomicin and MG132, with the effect greatest in the T1A cell line which has partially reduced chymotryptic activity.

However, we did not see a significant increase in soluble tau levels in T1A cells although there was a significant accumulation of tau in cells treated with MG132 (Figure 4.15A & 4.16A). The effect of MG132 on tau levels within the cells could not be accounted for by its impact on the three proteolytic activities of the proteasome (Figure 4.12-14). Although cells treated with epoxomicin and MG132 displayed comparable levels of chymotryptic and caspase-like activity, we did not see a similar increase in soluble tau levels in the cells that were treated with epoxomicin (Figure 4.16A). There were no significant changes in the tryptic activity (Figure 4.13) in mock, wild-type, and mutant T1A SH-SY5Y cells treated with MG132 compared to each respective cell lines under no treatment, suggesting the accumulation of tau found in cells treated with MG132 is not due to changes in any of the three proteolytic activities of the proteasome. We also did not find any changes in the expression of endogenous proteasome beta subunit 2 (PSMβ2) (Figure 4.15A & 4.17), which indicate no detectable changes in proteasome complex expression. Therefore, the accumulation of soluble tau in cells treated with MG132 may be attributed to changes in the level of other intracellular proteases by MG132 treatment, such as calpain. MG132 has been found to be a potent inhibitor of calpain (Kisselev et al, 2001) and other research groups have found a role in calpain in mediating tau turnover (Johnson et al., 1989; Xie & Johnson, 1998; Park & Ferreira, 2005).

Although we found an accumulation of soluble tau with MG132 treatment, we found a opposing effect in T1A cells treated with \( \text{H}_2\text{O}_2 \) compared to untreated mock cells (p<0.05), although the decrease in tau levels was not significantly different from untreated T1A cells (Figure 4.15A&4.16A). This is contrary to what we found in our control experiments where we observed an increase in tau levels with \( \text{H}_2\text{O}_2 \) treatment (Figure 4.11). However, our control experiment
was done on cells at a higher passage number and previous studies done in our laboratory found a decline in the expression of the pWPI expression vector as infected SH-SY5Y cells are passaged, which may contribute to the difference in tau levels under similar treatment conditions (Figure 4.18). Expression of the His\textsubscript{6} affinity tag as seen in Figure 4.15A ensures that the cells used in the current experiment is expressing the pWPI-WT and -T1A mutant β5 vectors.

When we examined whether MG132 and H\textsubscript{2}O\textsubscript{2} had a similar effect on insoluble tau as what we found for soluble tau, there was a significant decrease in NP40-insoluble tau in cells exposed to oxidative stress (Figure 4.16). Exposure to oxidative stress by H\textsubscript{2}O\textsubscript{2} treatment may increase the solubility of endogenous tau in SH-SY5Y cells, promoting the degradation of soluble tau and hindering its aggregation into insoluble inclusions. We also found that chronic inhibition of the chymotryptic activity in mutant T1A cells contributed to significantly less insoluble tau than mock and wild-type under all treatment conditions (p<0.05), although we did not see any significant increases in the level of tau in the soluble fraction (Figure 4.16). Further analysis must be done to determine why chronic impairment of the β5 subunit affects insoluble tau without major effects on soluble tau. There were no differences in NP40-insoluble tau in cells treated with MG132 compared to the other treatment conditions despite the significant accumulation of soluble tau (Figure 4.16), suggesting that the effects of MG132 is involved in the turnover of soluble, but not insoluble, tau.
Figure 4.16: Significant increase in soluble tau expression in cells treated with MG132 and significant decrease in insoluble tau in T1A cells, with effects most prominent in cells exposed to oxidative stress.
SH-SY5Y neuroblastoma cells infected with the mock (M), WT, and T1A β5 mutant lentivector was differentiated for 7 days with media containing 1% FBS and 10uM RA. The cells were subsequently treated with 25 nM epoxomicin for 24 hrs, 0.5 uM MG132 for 24 hrs, or 0.5 mM H2O2 for 1 hr with a 24 hr recovery period in differentiation media. Cells were harvested using standard protocol and 30 ug of protein was run on western blot. NP-40 insoluble pellets were subsequently lysed in 2% SDS/6M urea buffer then run on western blot. Western blot was probed for tau and GAPDH as loading control (Figure 4.15A). Densitometric analysis of differentiated mock cells exposed to no additional treatment was used as baseline and relative protein levels was plotted against cell type and treatment condition. Values are means ± SEM. * denotes p<0.05, ** denotes p<0.01, and *** denotes p<0.001 between mocked untreated cells to each experimental condition by two way ANOVA test and Bonferroni multiple comparison post-test (n=5-7).
Figure 4.17: No significant changes in proteasome beta subunit 2 expression with genetic or pharmacological inhibition of the proteasome.

SH-SY5Y neuroblastoma cells infected with the pWPI vector were differentiated with reduced serum and RA for 7 days. The cells were then treated with 25 nM epoxomicin for 24 hrs, 0.5 uM MG132 for 24 hrs, or 0.5 mM H₂O₂ for 1hr with a 24 hr recovery period in differentiation media. Cells were harvested using standard protocol and 30ug of protein was run on western blot. Western blot was probed for PSMβ2 and GAPDH as loading control (Figure 4.15A). Densitometric analysis of differentiated mock cells exposed to no additional treatment was used as baseline and relative protein expression was plotted against cell type and treatment condition. Values are means ± SEM. Significance was analyzed between mocked untreated cells to each experimental condition by two way ANOVA test and Bonferroni multiple comparison post-test (n=7).
Figure 4.18: Decline in pWPI vector expression in SH-SY5Y cells with increasing passage number.

SH-SY5Y neuroblastoma cells were infected with the pWPI empty vector (M), or pWPI-β5 WT or β5 T1A mutant vector. The cells were maintained in culture medium containing 10% FBS and harvested using standard protocol after passage 1, 10 and 15. The lysates were tested for levels of chymotryptic activity using the proteasome activity assay and for vector expression on western blot. Thirty microgram of protein was separated on SDS-PAGE and probed for expression of the His$_6$ affinity tag with tetra-his antibody (1:1000) and GAPDH (1:5000) as loading control. Inhibition of chymotryptic activity by expression of the mutant β5 subunit in T1A cells declines with increasing passage number as expression of the β5 mutant vector is loss over time.
5 Discussion

Alzheimer’s disease (AD) is the leading cause of dementia, accounting for over 60% of all dementia cases (Alzheimer’s Association, 2011). Brains of AD patients display protein aggregates in the form of senile plaques and neurofibrillary tangles, composed of amyloid-β and tau, respectively, within the brain. The accumulation and aggregation of amyloid-β and tau contributes to the progressive loss of memory and cognitive ability experienced by those affected with AD. Therefore, impairment of proper turnover of these two proteins has been implicated in the development and progression of AD.

The major protein degradation pathway in eukaryotic cells is the ubiquitin-proteasome system, which is responsible for the degradation of 80-90% of all proteins within the cell (reviewed by Sorokin et al., 2009). Disruption of this degradation system therefore contributes to substantial accumulation of unwanted or damaged proteins within the cell and its impairment has indeed been implicated in a number of neurodegenerative diseases (Keller et al., 2000; reviewed by Ciechanover & Brundin, 2003).

Several lines of evidence suggest a role of proteasome inhibition in AD. The proteolytic activity of the proteasome has been found to decline with age (reviewed by Friguet et al., 2000 & Ding et al., 2006), correlating with the increased risk of AD with age. Specifically, Keller and colleagues (2000) found a significant decrease in proteasome activity within the brain of AD patients, with the effect being especially prominent in vulnerable brain regions of AD. Also, protein aggregates found within the brains of AD patients are often heavily ubiquitinated, indicating impairment in the degradation of ubiquitinated proteins designated for the proteasome (reviewed by Mayer, 2003).

In addition to biochemical evidence supporting a role of the ubiquitin proteasome system in AD, mutations in genes encoding ubiquitin have also been found in patients of AD. Mutant forms of the polyubiquitin B (UBB) gene have been found in patients of AD and other neurodegenerative diseases (reviewed by van Leeuwen et al., 2006). Mutation of the UBB gene impedes the breakdown of polyubiquitin chains used to tag proteins for proteasome degradation, thereby delaying degradation of proteins through the ubiquitin proteasome pathway (Lam et al., 2000).
Gene mutations of the ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) gene have also been implicated as major contributor to AD pathogenesis due to UCH-L1’s role in synaptic signalling and recycling of monoubiquitin peptides for use within the cell. Impairment of proper UCH-L1 function has been found to reduce proteasome activity and promote protein accumulation and aggregation (Castegna et al., 2002).

With a strong case for decreased proteasome activity contributing to AD pathology, we investigated the role of impaired proteasome function in the level of endogenous tau in a cell culture model. Specifically, we looked at changes in tau levels as tau accumulation and neurofibrillary tangle formation have been found to correlate with the level of dementia in patients of AD (Arriagada et al., 1992; Santacruz et al., 2005; Oddo et al., 2006; Hansson et al., 2006). To the best of our knowledge, there have been no studies done to investigate the effects of chronic proteasome inhibition on tau protein levels in a cell culture or animal model. Therefore, we aimed to develop a cell culture model that chronically impairs proteasome activity through genetic mutation of a proteolytic subunit of the proteasome rather than through pharmacological inhibition alone, which we believe would generate a more specific and representative model of AD disease progression.

5.1 Genetic Mutation of the β5 Proteasome Subunit through Stable Lentiviral Infection of SH-SY5Y cells

Since chymotryptic degradation is considered the rate determining step in proteasome degradation (Kisselev et al., 1999) and it has been found to be decreased by ~30-50% in different brain regions of Alzheimer’s disease patients (Keller et al., 2000), we developed a cell culture model in SH-SY5Y neuroblastoma cells that specifically impairs the chymotryptic activity of the proteasome. The chymotryptic activity of the proteasome is mediated by the β5 subunit and expression of the Thr1 to Ala (T1A) mutant form of β5 was found to significantly inhibit chymotryptic activity of the proteasome (Li et al., 2004). Using a similar approach, we introduced the T1A mutation into the human wildtype (WT) β5 cDNA by site-directed mutagenesis to create the mutant T1A β5 construct. This construct was then inserted into three different vectors for expression in SH-SY5Y cells: TR/pTREhyg, pLVCT-tTR-KRAB, and
pWPI (Figure 4.2). We initially wanted to express our mutant T1A construct in an inducible system as it allows for tighter and more specific regulation on gene expression; however, expression of both our inducible plasmids, pTREhyg and pLVCT-tTR-KRAB, were unsuccessful. Previously, our laboratory was able to establish an inducible cell line using the pTREhyg plasmid in HEK293 cells by Lipofectamine 2000 transfection. Unfortunately, multiple attempts using Lipofectamine 2000 and the electroporation Nucleofector Kit, which boasts up to 70% transfection efficiency, all failed (Figure 4.3) in SH-SY5Y neuroblastoma cells. This may be in part due to the low transfection efficiency of SH-SY5Y cells (Martin-Montanez et al., 2010). Therefore, we moved on to using a lentiviral delivery system in hopes of increasing the infection efficiency and establishing long-term stable expression of our vector construct. The major drawback to using the inducible lentiviral pLVCT-tTR-KRAB plasmid was the inability to select for successful clones. The plasmid does not carry an antibiotic resistance gene for selection and the infection rate was very low (Figure 4.4). Although we were able to visualize several clones through fluorescence microscopy, the clones were so few in number that we were not able to detect any expression of our construct using SDS-PAGE (Figure 4.4B).

In the end, we opted to use the pWPI stable expression lentiviral system. This lentiviral system has a very high infection rate and contains a neomycin resistance gene for selection of successful clones. After only two days post-infection, we were able to detect the exogenously expressed β5 gene by probing for the His6 epitope tag by western blotting (Figure 4.5). In addition to expression of the wildtype β5 and mutant T1A β5 subunit, the β5 subunits must also be incorporated into proteasome complexes to induce any changes in the proteolytic activities of the proteasome. Separation on a glycerol density gradient revealed incorporation and maturation of the wildtype β5 and mutant T1A β5 into proteasome complexes that co-eluted with endogenous proteasome subunit β2 (Figure 4.6). Since maturation of the β subunits only occur after proper incorporation into proteasome complexes (Schmidtke et al., 1996), the fact that most of the wildtype β5 and mutant T1A β5 subunits detected by probing for the His6 affinity tag were in its mature form indicate that the exogenous β5 proteins were incorporated into proteasome complexes within the cell. Expression of the wildtype β5 and mutant T1A β5 subunits in complexes that co-eluted with ≥400kDa markers, corresponding to half and full proteasome complexes (α7β7 and α7β7β7α7, respectively), and co-expression of endogenous β2 subunits within the same fractions further confirm that our β5 vectors are incorporated and
matured into proteasome complexes.

Since complete knockout of the β5 subunit was found to be lethal in various model systems (Heinemeyer et al., 1993; Belote & Fortier, 2002), our model system impairs proteasome function by incorporation of non-functional β5 subunits into the proteasome, with the expression of endogenous β5 subunits still intact. We did not investigate the relative levels of exogenous β5 subunit incorporation compared to endogenous β5 into proteasome complexes as there is no reliable commercially-available antibody that effectively recognizes endogenous β5 subunits. However, we did determine the effect of the T1A β5 mutant on overall proteolytic activity of the proteasome. We found that expression of mutant β5 was able to reduce the chymotryptic activity of the proteasome by about 40% (Figure 4.7), a level comparable to that found in the brains of AD patients (Keller et al., 2000). There was no significant increase in chymotryptic activity in cells overexpressing the wildtype β5 subunit, which suggests that expression of the wildtype β5 and mutant T1A β5 subunit may not affect the number of proteasome complexes formed within the cell; rather, the exogenous subunits expressed by our vector compete with endogenous β5 subunits in forming proteasomes. This notion is supported by the fact that we did not detect any changes in β2 subunit expression between mock, wildtype β5 and T1A β5 mutant cell lines (Figure 4.17), suggesting no increase in proteasome formation. To further examine changes in endogenous proteasome levels, future experiments can be done to isolate and identifying the expression of proteasomes by western blot analysis.

Also, although we do not have evidence that indicate the expression of exogenous β5 may affect the maturation of β5 subunits or incorporation of other proteasome subunits into full proteasome complexes, future experiments can be conducted with β5 subunits tagged with different length epitope tags and examine for changes in proteasome maturation and assembly.

5.2 Differentiation of SH-SY5Y Neuroblastoma Cells to Promote Neuronal Phenotype

Although undifferentiated SH-SY5Y neuroblastoma cells are one of the most commonly used
neuronal-like cell lines in neuroscience research, they lack several features of mature neurons. Differentiation was found to induce the expression of neuron specific markers, such as NeuN and tau, and promote a more neuronal morphology (Agholme et al., 2010). Differentiated SH-SY5Y cells were found to more closely resemble mature human neurons on both a biochemical and morphological level by increased expression of neuron specific markers and extension of neurites (Agholme et al., 2010). Thus, to establish a more neuronal phenotype in our infected SH-SY5Y cells, we treated them with commonly-used differentiation factors: nerve growth factor (NGF) and retinoic acid (RA), for 6-10 days (Figure 4.8). We found that treatment with RA in reduced serum media induced the highest level of tau and a neuron-like morphology in the neuroblastoma cells with substantial elongation and branching of neurites (Figure 4.9). Increasing expression of endogenous tau by differentiation rather than overexpressing recombinant tau through transfection procedures avoids some issues involved in the use of recombinant tau such alternation of protein-protein interactions within the cell (David et al., 2002).

5.3 Soluble Tau Expression is Not Mediated by Chymotryptic Degradation by the Proteasome

In this study, we did not see any significant changes in soluble tau levels following chronic inhibition of the chymotryptic activity of the proteasome (Figure 4.16). Under basal conditions, chymotryptic activity of the proteasome was decreased by ~40% in β5 mutant T1A cells compared to control (Figure 4.12) but this level of activity was not sufficient to elicit any changes in the levels of ubiquitin or soluble tau (Figure 4.15B& 4.16A). A 40% decrease in chymotryptic activity may not have been sufficient to induce an accumulation of ubiquitin within the cell or proteolytic activities of the other two active sites of the proteasome may have acted as a compensatory response to degrade ubiquitinated proteins typically targeted for β5 degradation. Previous results from our lab and others (Li et al., 2004) also found no gross differences in cell viability or ubiquitinated immunoreactivity when comparing T1A β5 mutant cells to wildtype β5 or control cells under basal conditions. Differences between wildtype β5 and T1A β5 mutant cell lines only became evident after exposure to cadmium, which induces oxidative stress (Li et al.,
An accumulation of ubiquitinated proteins was found in β5 mutant cells but not β5 WT cells following 6μM cadmium treatment, suggesting either an increased sensitivity of β5 mutant cells to cadmium-induced oxidative stress or a reduced capacity to clear oxidized proteins. Previous results from our laboratory also found an H$_2$O$_2$-induced increase in ubiquitin immunoreactivity in HEK293 cells expressing T1A β5 mutant cells compared to control and wildtype β5 cells (D’Souza & Tandon, unpublished). However, the present study did not find an accumulation of ubiquitinated proteins in T1A β5 mutant cells at our treatment concentration of 0.5mM H$_2$O$_2$. It is possible the different cell lines, HEK293 human embryonic kidney cells versus differentiated SH-SY5Y cells, and the higher treatment concentration of H$_2$O$_2$ previously may account for the differences in ubiquitin accumulation.

We also did not see any changes in soluble tau levels in cells treated with epoxomicin, indicating that even at < 10% chymotryptic activity compared to control (Figure 4.12), there were no significant effects on tau (Figure 4.16A). The significant accumulation of ubiquitinated proteins suggests degradation of ubiquitinated proteins targeted for the proteasome was in fact inhibited. Contrary to our hypothesis, our results suggest endogenous tau may not be degraded by the chymotryptic activity of the proteasome because inhibition of chymotryptic activity did not result in accumulation of tau within the cell. This is in line with a study done by Cardozo and Michaud (2002) where inhibition of the chymotryptic activity of the proteasome had little effect on the rate or pattern of isolated tau degradation. Although inhibition of β5 activity did not affect overall levels of tau, it would be interesting to investigate whether impaired chymotryptic activity affects the accumulation of truncated or phosphorylated tau species within the cell in future studies.

5.4 Expression of a Catalytically Inactive β5 Proteasome Subunit Mutant Significantly Decreased Insoluble Tau Expression

Although inhibition of proteasome chymotryptic activity did not result in any changes in soluble tau levels in our cell culture model, we found a significant decrease in NP-40 insoluble tau in the T1A β5 mutant cell line under all treatment conditions compared to mock and wildtype β5 cells (Figure 4.16B). There was significantly less insoluble tau in T1A β5 mutant cells under basal
conditions, with no significant differences in cells exposed to the additional treatment of epoxomicin or MG132. Therefore, the decrease in insoluble tau seemed not to be correlated with the level of chymotryptic inhibition but rather with the chronic inhibition of β5 activity.

In our study, T1A β5 mutant cells and cells treated with H₂O₂ both showed a significant decrease of NP-40 insoluble tau compared to control. Chronic inhibition of the proteasome with pharmacological agents was found to contribute to increased protein oxidation within SH-SY5Y cells (Ding et al., 2004). Therefore, mutation of the β5 subunit may contribute to an accumulation of oxidized proteins by impaired ability to remove oxidized proteins within the cell, resulting in exposure to mild oxidative stress in T1A β5 mutant cells.

The level of oxidative stress in T1A β5 mutant cells and cells treated with H₂O₂ at 0.5mM for 1hr is likely mild rather than severe as there were no signs of cell death or accumulation of ubiquitin from proteasome inhibition induced by heavy accumulation of oxidized proteins. We speculate that the decrease in insoluble tau in T1A β5 mutant cells and cells exposed to H₂O₂ may be due to 1) unfolding of proteins from exposure to mild oxidative stress, providing better access through the proteasome core, promoting its degradation; 2) increased solubility of proteins from mild oxidative stress, resulting in a decrease in tau aggregate formation. Oxidative stress has been found to induce unfolding of tertiary protein structure (reviewed by Ding et al., 2006).

Chronic inhibition of the β5 subunit may also modify the activity level of other catalytic subunits of the proteasome. Kisselev and colleagues (1999 & 2006) found that the inhibition of one catalytic site can affect the activity at other proteolytic sites. Chronic inhibition of any specific proteolytic activity of the proteasome will likely induce the activity of the other two proteasome subunits to maintain overall protein degradation within the cell. We did see a trend in increased trypsic activity, but not caspase-like activity, in T1A β5 mutant cells compared to mock infected SH-SY5Y cells (Figure 4.13 & 4.14) but this was not found to be significant. Trypsin-like activity may contribute to increased degradation of soluble tau, resulting in less tau in the soluble pool available for the formation of aggregates, but this remains to be confirmed.
5.5 Higher Level of Proteasome Tryptic Activity in Mutant T1A β5 Mutant Cells May Contribute to Soluble Tau Degradation

We saw a trend in increased tryptic activity in mutant β5 T1A cells compared to mock under all treatment conditions although the increase in trypsin-like activity did not reach statistical significance (Figure 4.13). The results may not have approached significance due in partly from the lower signal-to-noise ratio of the Boc-LRR-AMC fluorogenic substrate used for assessment of tryptic activity compared to the Suc-LLVY-AMC substrate used for chymotryptic activity (O’Brien et al., 2006). Although we controlled for background activity of the proteasome, we were unable to control for all of the other proteases that may also be involved in the cleavage of the Boc-LRR-AMC substrate, which may contribute to the large variation in our assay (Figure 4.13). Unfortunately, the most widely accepted technique for measuring proteasome activity is still with assays using fluorogenic substrates and so, although our results were unable to reach significance, we should still consider the possibility of an increase in tryptic activity in T1A β5 mutant cells compared to mock cells as our observations may be hampered by the inadequacies of the current experimental techniques available.

To the best of our knowledge, we are the first study to chronically impair the chymotryptic activity of the proteasome by genetic means and look at its effects on proteasome activity at all three active sites of the proteasome, there are no past studies to support whether chronic impairment of β5 activity results in upregulation of the tryptic or caspase-like activity. A study has shown a concurrent decrease in both chymotryptic and caspase-like activity of the proteasome in AD patients but the decrease in β1 activity was not a direct result of impaired β5 activity (Keller et al., 2000). Our study shows that the trend in increased tryptic activity in T1A β5 mutant cells is likely due to an induction of β2 activity rather than an increase in β2 subunit expression as we do not see a significant increase in β2 expression in T1A β5 mutant cells compared to mock infected SH-SY5Y cells under all treatment conditions (Figure 4.17). Further research must be done to confirm what effects chronic impairment of chymotryptic activity may have on the other proteolytic sites of the proteasome.
Although not reaching statistical significance, we also saw a correlating trend in increased tryptic activity with decreased levels of soluble tau (Figure 4.13 & 4.16A). To our knowledge, only one study has looked at the direct relationship between β2 activity of the proteasome and tau. Cardozo and Michaud (2002) suggest that tau degradation may be mediated by tryptic activity of the proteasome as their studies show that inhibition of tryptic activity by leupeptin reduced tau degradation by 40% while inhibition of chymotryptic activity had no significant effect on the degradation rate or cleavage pattern of tau.

Also in support of a role of tryptic activity in the degradation of tau, the β2 subunit of the proteasome primarily cleaves after basic residues and tau has a basic isoelectric point of pI 6.5-8.5 (Ksiezek-Reding et al, 1988) due to its abundance in basic amino acids (Mukrasch et al., 2009). Therefore, under basal conditions, tau cleavage may be mediated by the tryptic activity of the proteasome. However, common post-translational modifications of tau, such as hyperphosphorylation as seen in Alzheimer’s disease, results in a more acidic form of tau with a pI of 4.8-6.6 (Greenberg & Davies, 1990), raising the possibility of an involvement of proteasome caspase-like activity in the cleavage of tau. While inhibition of the tryptic activity alone resulted in a 40% decrease in tau degradation, inhibition of both tryptic and caspase-like activity of the proteasome inhibited tau degradation by over 90% (Cardozo et al., 2002).

Many of the studies done involving proteasome inhibition and tau degradation make use of lactacystin, epoxomicin, or MG132 for pharmacological inhibition of the proteasome (Grune et al., 2010; Liu et al., 2008; David et al., 2002; Feuillette et al., 2005). However, all three of these inhibitors inhibit all three activities of the proteasome, albeit at different rates and concentrations (Fenteany et al., 1995; Kisselev & Goldberg, 2001; Berkers et al., 2005). Lactacystin and MG132 have also been found to react with other proteases, such as cathepsins and calpains (Kisselev & Goldberg, 2001). Therefore, it is difficult to decipher which proteolytic pathway is actually involved in the degradation of tau while making use of these substrates. Epoxomicin is a more specific inhibitor of the proteasome with no other targets within the cell but studies done with epoxomicin have found no effects on tau degradation at lower concentrations (Hamano et al., 2009). Accumulation of ubiquitin and tau were detected at higher concentrations, in which case other activities of the proteasome may also be inhibited (Hamano et al., 2009), while a study done by Feuillette and colleagues (2005) also found no change in tau levels with low-
Increasing treatment concentrations of epoxomicin were not found to be correlated with the level of tau (Feuillette et al., 2005). Therefore, contrary to our hypothesis, it seems that the evidence to date seem to support a possible role of tryptic and caspase-like activity in the degradation of normal tau, with a lesser involvement of the chymotryptic activity of the proteasome. Since tau is highly modified in brains of Alzheimer’s patients, more research much be done to confirm which of the three proteosomal activities are most important in the degradation and turnover of normal tau and modified tau as seen in AD.

5.6 Inhibition of Proteasome Caspase Activity by 50% is Insufficient to Affect Soluble Tau Expression

As mentioned above, Cardozo and colleagues (2002) found that inhibition of tryptic and caspase-like activity of the proteasome reduced tau degradation by over 90% but inhibition of caspase-like activity alone displayed a marginal effect on tau degradation. Therefore, caspase-like degradation of tau may act as an alternative pathway for tau degradation, active primarily when other pathways become compromised. Experiments using yeast suggest a minor role of caspase-like proteolysis in the overall degradative activity of the proteasome: mutants lacking caspase-like activity showed little effect on overall protein degradation rate of the proteasome and no effects on cell viability (Heinemeyer et al., 1997). However, research done in eukaryotes suggest that the proteasomal caspase-like activity may have evolved to play more important roles in protein cleavage and allosteric regulation of other proteolytic sites of the proteasome (Kisselev et al., 1999).

In our study, treatment with epoxomicin reduced caspase-like activity to ~40% of untreated mock cells but did not result in any significant changes in soluble or insoluble tau levels (Figure 4.14 & 4.16); thus, either inhibition of β1-mediated caspase-like degradation by 60% is insufficient to elicit any major changes in the level of tau or tau is not a specific target of β1 activity. In agreement with previous results by Cardozo and colleagues (2002), our results suggest tau degradation may be mostly dependent on the tryptic activity of the proteasome as
inhibition of the chymotryptic activity by ~90% and caspase-like activity by ~60% in cells treated with epoxomicin resulted in no major changes in tau protein.

On the other hand, cells treated with MG132 displayed a significant accumulation of soluble tau though they had a similar proteasome activity profile to cells treated with epoxomicin (Figure 4.12-14). Therefore, it is likely that the accumulation of tau in cells treated with MG132 is not solely due to inhibition of proteasome activity, but rather to effects of MG132 on other proteases within the cell, such as calpain (reviewed by Myung et al., 2001).

5.7 Inhibition of Calpain Activity Contributes to an Accumulation of Soluble Tau

In our study, cells treated with MG132 displayed a significant accumulation of soluble tau and ubiquitinated proteins. There was an over four-fold increase in ubiquitin immunoreactivity and almost two-fold increase in soluble tau in cells treated with MG132 (Figure 4.15 & 4.16A). Cells treated with epoxomicin also exhibited an increase in ubiquitin accumulation compared to MG132-treated cells but not a similar increase in soluble tau levels. Cells receiving either treatments displayed similar proteasome activity profiles (Figure 4.12-14), but only cells treated with MG132 displayed the increase in soluble tau. Epoxomicin is an irreversible proteasome inhibitor without any other known targets within the cell while MG132 is a reversible inhibitor that is also known to inhibit other proteases in the cell, including calpain and cathepsin (reviewed by Myung et al., 2001). Therefore, the effects on tau levels we found in cells treated with MG132 may not be the result of proteasome inhibition but rather through interaction with other proteases within the cell.

Of particular interest is the role of calpain in tau turnover since calpain, but not cathepsin D, was found to produce tau fragments resembling those found in post-mortem Alzheimer brain (Mercken et al., 1995). Several other studies on the role of calpain proteolysis of tau have been conflicting. Johnson’s group supports the notion that calpain is a major mediator of tau degradation while protein kinase A promotes phosphorylation of tau and attenuates calpain activity, which is consistent with the accumulation of hyperphosphorylated tau seen in the brains
Proteasome inhibition was also found to promote calpain accumulation, which resulted in the degradation of normal tau within cells (Delobel et al., 2004). Subsequent studies went on to find that degradation of tau by calpain resulted in a 17 kDa tau fragment found in AD transgenic mouse models (Roberson et al., 2007) and overexpression of this tau fragment resulted in cell death and apoptosis in cells (Park and Ferriera, 2005), raising the possibility that calpain activity may induce tau pathology in AD. In addition to tau being a substrate of calpain in vitro, the decrease in calpain expression and activity correlated with the level of neuropathology in brains of AD patients (Nilsson et al., 1990; Ferreira & Bigio, 2011). Our results also support the notion that calpain is a mediator of tau turnover as treatment with MG132, an inhibitor of calpain activity (reviewed by Myung et al., 2001), resulted in almost twice normal tau expression (Figure 4.16A), a change that cannot be explained by changes in proteasome activity (Figure 4.12-14). The effects of MG132 on calpain inhibition represents a testable model for the decrease in calpain activity in AD patients, and this in turn may contribute to an accumulation of tau, leading to the formation of paired helical filaments and neurofibrillary tangles.

However, subsequent studies indicate that calpain may not be involved in the neuropathology found in AD patients. Garg and colleagues (2011) supports the role of calpain in the cleavage of tau, resulting in the formation of a 17kDa tau fragment but suggested that the neurodegeneration observed in AD mouse models may not be a result of the 17 kDa tau fragment derived from calpain cleavage (Garg et al., 2011).

Park & Ferreira (2005) found that transfection of the recombinant 17kDa tau fragment into CHO cells and hippocampal neurons resulted in significant degeneration and higher level of positive staining with TUNEL+ compared to control. Using a similar approach, transfections with two isoforms of the 17 kDa tau fragment by Garg’s group into N2a cells, CHO cells or hippocampal neurons did not induce cytotoxicity and the presence of the tau fragment was found in the brain of AD patients and of controls (Garg et al., 2011). The conversing results of the two groups could not be accounted for by their experimental approach as both groups used similar techniques: overexpression of recombinant full length and 17kDa tau in hippocampal neuron culture to investigate the effect of 17kDa tau on cytotoxicity and tau aggregation, analysis of full
length and 17kDa tau expression in brains of AD patients versus control, and use of calpain inhibitor to reverse the effects of calpain on tau (Park & Ferreira, 2005; Garg et al., 2011).

Although most of the evidence to date supports a role of calpain in tau degradation (Johnson et al., 1989; Litersky & Johnson, 1992; Park & Ferreira, 2005; Garg et al., 2011), a study contests with this finding. Brown and colleagues (2005) established that treatment of SH-SY5Y cells and primary neurons with calpain inhibitor MDL28170 did not alter tau expression, leading them to suggest that calpain may not be involved in tau degradation. The differences found in this study could not be explained by a difference in cell type or calpain inhibitor used as Brown, Ferreira and Garg’s group all made use of primary neuron cultures while both Brown’s group and Ferreira’s group made use of the same calpain inhibitor, MDL28170 (Brown et al., 2005; Park & Ferreira, 2005; Garg et al., 2011).

Calpain degradation of proteins within the cell is dependent on the tertiary conformation of the target protein (Cuerrier et al., 2005). Modifications of tau as seen in patients with AD, such as hyperphosphorylation and aggregation of tau, reduce the affinity of tau proteins for calpain degradation. Therefore, our results support a role of calpain in the degradation of normal tau but the role of calpain in mediating tau neuropathology requires further investigation.

5.8 Mutant β5 T1A Cells are More Vulnerable to Oxidative Stress

Oxidative stress has been implicated in the initiation and progression of a number of neurodegenerative diseases, including Alzheimer’s disease. At high and prolonged exposure, it has also been implicated in inducing aggregate formation (Davies, 2001) as well as inhibiting proper proteasome activity (reviewed by Ding & Keller, 2003). Without proper proteasome function, the cell is unable to cope with environmental stresses, resulting in a buildup of oxidative species and unwanted proteins within the cell. The presence of oxidized proteins in the cell also impedes the proper function of enzymes required for basic cell function. Therefore, we wanted to investigate the effects of oxidative stress on proteasome function, and how this may affect tau levels in a cell culture model.
Previous studies done in our lab found that although chronic proteasome impairment does not affect cell viability or accumulation of ubiquitin within the cell under basal conditions, additional exposure to oxidative stress resulted in increased signs of cell death and accumulation of protein carbonyls and ubiquitin in T1A β5 mutant cells compared to control (D’Souza & Tandon, unpublished). To investigate whether oxidative stress induced-vulnerability of T1A β5 mutant cells affects tau levels in cells, we exposed the cells to oxidative stress by treatment with \( H_2O_2 \). Hydrogen peroxide treatment resulted in a significant decrease in chymotryptic activity within the cell, with little to no change in tryptic or caspase-like activity (Figure 4.12-14). Inhibition of chymotryptic activity by \( \sim 40-60\% \) by the T1A β5 mutation was insufficient to induce any accumulation of ubiquitinated proteins within the cell, which is in line with previous results from our lab and that of Li and colleagues (2004). Significant increase in ubiquitin immunoreactivity was only detectable in HEK293 cells exposed to oxidative stress at \( H_2O_2 \) concentrations double that used in the current study (D’Souza & Tandon, unpublished data). Further, changes in cell viability were only evident when cells were incubated in 10 times the concentration of \( H_2O_2 \). Therefore, although the cells used in the current experiment may have displayed more prominent changes in proteasome activity or increased ubiquitin accumulation at higher exposure to \( H_2O_2 \), we did not incubate the cells in higher levels of oxidative stress because differentiation of SH-SY5Y neuroblastoma cells with retinoic acid sensitized the cells to \( H_2O_2 \), with signs of cell death at concentrations of \( H_2O_2 \) higher than 0.5mM.

When we investigated the effects of tau levels in response to oxidative stress, we found a significant decrease in soluble tau in T1A β5 mutant cells exposed to \( H_2O_2 \), but not in wildtype β5 cells exposed to the same level of oxidative stress (Figure 4.16A). This indicates that although chronic proteasome inhibition or \( H_2O_2 \) treatment alone did not induce any changes in soluble tau levels, the combined effect of both were able to elicit a significant decrease in soluble tau. Impaired chymotryptic activity of the proteasome by \( 40\% \) may impede the cells ability to remove oxidized proteins within the cell, thereby reducing the cells ability to recover from oxidative stress and increasing the basal level of oxidized proteins within the cell. With the reduced ability to cope with oxidative stress in T1A β5 mutant cells, pharmacological treatment with 0.5mM \( H_2O_2 \) would result in a sufficient build up of oxidized proteins within the cell to promote the unfolding of tau. This would result in a greater affinity of tau for degradation through the
proteasome or by other proteases within the cell and result in a decrease in soluble tau levels seen in T1A β5 cells exposed to oxidative stress. This is in line with the literature where mild oxidative stress has been found to promote conformational changes in target protein for easier translocation through the 20S proteasome (reviewed by Ding & Keller, 2001).

Based on the same rationale, we speculate that the significant decrease in NP-40-insoluble tau found in T1A β5 mutant cells and cells treated with H$_2$O$_2$ (Section 5.4) is due the effects of impaired chymotryptic activity of the proteasome and exposure to 0.5mM of H$_2$O$_2$ on the accumulation of reactive oxygen species within the cell, leading to an increase in unfolding and degradation of tau proteins and decrease in insoluble tau levels.
6 Conclusion

Taking all of the evidence presented thus far, we believe that turnover of endogenous tau is not mediated by the chymotryptic activity of the proteasome. The tertiary structure of tau and its abundance of basic residues is consistent with a key role of trypsin-like activity of the proteasome as well as other proteases, such as calpain, in its degradation. It is also possible that only certain tau species within the cell may be degraded through the ubiquitin proteasome system while posttranslational changes to tau may direct it down a proteasome-independent pathway of degradation.

We established a cell culture system that expressed an inactive mutant form of the β5 subunit of the proteasome to mimic the chronic age-related impairment of proteasome function seen in aging individuals and those with neurodegenerative diseases such as AD. Proteasome inhibition by genetic mutation of the proteasome subunit is a preferable means of targeting proteasome activity as treatment with pharmacological agents can affect other pathways within the cell not specific to the proteasome such as the effects of MG132 on inhibiting calpain (reviewed by Myung et al., 2001).

Proteasome inhibition impedes the cell’s ability to clear damaged and unwanted proteins from the cell, and we wanted to investigate the role of oxidative stress and proteasome inhibition on the accumulation of tau within the cell. We found that chronic impairment of the proteasome sensitized cells to the presence of reactive oxygen species. In our study, exposure to H$_2$O$_2$ in T1A mutant β5 cells exposed to mild oxidative stress displayed the greatest amount of tau degradation compared to mock and wildtype β5 cells, which is in line with evidence indicating an increase in proteasome activity with mild oxidative stress (reviewed by Keller et al., 2002). However, if we were to increase our H$_2$O$_2$ treatment concentration, we may have found the opposite effect. When oxidative stress becomes increasingly severe beyond the proteasome’s ability to compensate, the proteasome becomes overwhelmed with oxidized material and damaged proteins build up within the cell, significantly decreasing the proteolytic ability of the proteasome (reviewed by Ding & Keller, 2003). Severe oxidative stress promotes the aggregation of proteins that block access through the proteasome catalytic core, inhibiting its
activity, thereby promoting a further build up of reactive species within the cell. The effects of oxidative stress on proteasome inhibition result in a detrimental feedback loop that promotes protein aggregation and neuronal cell death. Therefore, although mild oxidative stress may promote the degradation of tau, exposure to higher levels of oxidative stress may sufficiently impedes proper proteasome function and promotes tau accumulation and aggregation within the cell. A representative diagram displaying the relationship between proteasome dysfunction, oxidative stress, and tau protein is seen below (Figure 5.1).

Figure 5.1: Schematic Representation of the Tau Degradation Pathway

There are several limitations to our study that should be kept in mind. As mentioned before, proteasome activity was measured using assays that measure fluorogenic substrate cleavage. This is the most common technique currently in use to assess proteasome activity but it has its limitations. Although the substrates are targeted for cleavage by the specific subunits of the proteasome, they may also be cleaved by other proteases within the cell. The ability of proteasome inhibitors to control for background and noise is also limited by the affinity of the
inhibitors for each proteolytic site of the proteasome as most proteasome inhibitors have high affinity for the chymotryptic activity of the proteasome and can only affect the other two proteolytic sites at much higher treatment concentrations (reviewed by Myung et al., 2001). Also, proteasome activity assays may not be truly representative of the proteasome activity in vivo as chaperones of proteasome activity, such as ATP, are not accounted for in an in vitro assay. Therefore, although the proteasome activity assays can give us an idea of proteasome cleavage within the cell, it is not an absolutely accurate representation of proteasome activity.

In our study, we investigated the effects of chymotryptic activity of the proteasome on tau protein only by establishing a vector expressing a mutant β5 subunit of the proteasome. Therefore, further studies must be done to definitively conclude how genetic mutation and inhibition of the β1 and β2 subunits may affect overall proteasome function and how it may affect normal and phosphorylated tau levels.

Also, due to the sensitivity of differentiated SH-SY5Y neuroblastoma cells to oxidative stress, we were unable to contrast the effects of mild and severe oxidative stress on proteasome function and tau protein levels in our experiment.

Lastly, it is important to bear in mind that although our study investigated the role the proteasome may play in mediating endogenous tau levels within the cell, other proteases and pathways may also be involved. This was highlighted when cells treated with MG132 displayed a significant accumulation of soluble tau in this study, although this could not be accounted for by the effects of MG132 on proteasome activity. The effect of MG132 on other non-proteasomal proteases within the cell, such as calpain, may have played a role in mediating tau levels within the cell. As tau becomes aggregated into inclusions and NFT, it may also be directed down the autophagy-lysosomal degradation pathway as this pathway is capable of engulfing and degrading large protein complexes that cannot pass through the proteasome catalytic core. Therefore, although the proteasome may be involved in the degradation of tau, other degradation pathways within the cell, such as calpain and the autophagy-lysosomal pathway, may also play significant roles in tau turnover.
7 Future Directions

7.1 Establish Genetic Mutations of β1 and β2 Subunits of the Proteasome

To investigate whether normal tau protein is mediated by tryptic- or caspase-like activity of the proteasome, it would be useful to develop vectors that express mutant forms of the β1 and β2 subunits. Genetic impairment of the tryptic- and caspase-like activities would result in a more stable, chronic and specific inhibitor of the proteasome, avoiding some key issues with the use of pharmacological agents to inhibit proteasome activity. This would establish a better system for investigating proteasome inhibition than the models used today.

7.2 Investigate the Effects of Proteasome Degradation on the Cleavage Pattern of Tau

Our study examined the effects of proteasome inhibition on the overall level of tau levels within SH-SY5Y neuroblastoma cells but did not examine the effects of impaired proteasome activity on the cleavage pattern of tau. Although proteasome inhibition did not seem to have a direct effect on tau levels in our study, impaired β1, β2, and β5 activity may have individual effects on the cleavage pattern of tau. By detecting for the generation of different tau fragments after inhibition of the three proteolytic sites of the proteasome, we can examine how each of the three proteasomal activities may contribute to the generation of truncated or toxic tau fragments.

7.3 Investigate the Effects of Chymotryptic Activity on Overall Proteasome Activity

Our study showed a trend in increased tryptic activity in cells expressing impaired chymotryptic activity. Further research can be done to investigate the inhibition of a specific proteasome subunit on the proteolytic activity of the other two subunits such as introducing a genetic mutation or knocking out the β1 or β2 subunit of the proteasome, then assaying the cells for
changes in proteasome activity. This will shed light on how the proteasome may mediate its own activity in response to environmental stresses or presence of certain substrates or enzymes.

7.4 Investigate the Effects of Hyperphosphorylation on Proteasome Degradation of Tau

Studies suggest that normal tau may be mediated by trypsin-like degradation of tau due to the abundance of basic residues on tau. Since hyperphosphorylation of tau brings tau to a more acidic isoelectric point, it would be interesting to see if there is a shift of increasing prominence in β1-mediated caspase-like activity of the proteasome with increased phosphorylation of tau. Keller and colleagues (2000) found a significant decrease in caspase-like activity of the proteasome in patients with AD. If hyperphosphorylation of tau is in fact degraded by the β1 subunit of the proteasome, the impairment in caspase-like degradation of tau would contribute to the accumulation of hyperphosphorylated tau in the brains of AD patient.


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