UNIVERSITY OF CALIFORNA SAN DIEGO

Lysosomal Leakage of Cathepsin B in Alzheimer's Disease and Related Neurodegenerative Brain Disorders

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

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by

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The thesis of Gen Ito is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

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Alzheimer's disease (AD) is a severe neurodegenerative disease that is becoming one of the major causes of death worldwide. One of the main indicators of AD is neuronal death due to the extracellular accumulation of amyloid-beta (Aβ) plaques, one form being Aβ42 peptide oligomers. Previous studies have shown the involvement of a lysosomal protease called cathepsin B (CatB) and lysosomal leakage, and its implications in cognitive deficits and neuropathology of AD and other related neurodegenerative diseases. These studies show the potential for CatB as a therapeutic target. Here, the extent to which Aβ42 oligomers induce

lysosomal leakage and the translocation of CatB is investigated. It is hypothesized that Aβ42 induces lysosomal leakage and the translocation of CatB to the cytosol associated with cell death. Through lactate dehydrogenase (LDH) assays, Aβ42 treatment of SK-N-MC and SH-SY5Y human neuroblastoma cells resulted in cell death cytotoxicity. Fluorescent imaging by acridine orange (AO) staining showed that in both SK-N-MC and SH-SY5Y cells, Aβ42 treatment induced lysosomal leakage. Moreover, western blot analysis of the cytosolic fractions of Aβ42-treated BV2 microglial cells obtained via sucrose density gradient centrifugation showed the presence of pro- and mature CatB. Lastly, using the LES12 bifunctional probe in Aβ42-treated SH-SY5Y cells revealed increased activity of cysteine cathepsins in a cytosolic neutral pH environment. These findings advance the understanding of lysosomal leakage in AD and other related neurodegenerative diseases, demonstrating the new finding that Aβ42 oligomers induce lysosomal leakage of CatB associated with cell death.

A. Introduction

Cathepsin B (CatB) has been shown to participate in Alzheimer's disease (AD) based on findings that CatB is elevated in AD patients, elevated CatB in serum of AD patients correlates with cognitive deficits (Hook et al., 2020), and knockout of the CatB gene in AD mouse models substantially improves memory deficits and reduces Aβ peptides (Kindey et al., 2012). These findings support CatB participation in severe memory deficits and the neuropathology of AD.

It will be important to understand the mechanism of CatB involvement in AD. Therefore, the question that will be addressed in this project is: Amyloid beta (Aβ)-induction of lysosomal leakage results in what extent of CatB translocation from the lysosome to the cytosol, participating in neuronal cell death? It is hypothesized that Aβ-induction of lysosomal leakage results in the translocation of CatB protein and activity from the lysosome to the cytosol associated with cell death. The goal of the project is to obtain insight in the involvement of cytosolic CatB in the neuropathology of AD and other related neurodegenerative diseases. This research will allow for new mechanistic understandings of Aβ42 oligomer accumulation and CatB lysosomal leakage involved in AD and related neurodegenerative diseases.

B. Alzheimer's disease: critical mechanisms and therapeutics issues to be addressed in the field

With the current fast-paced growth in the population, neurodegenerative diseases are increasingly becoming one of the major causes of death globally (GBD 2016 Neurology Collaborators, 2019). One of these diseases is AD. Just within the US population, it is said that 10 percent of people aged 65 and older has AD and this number only increases with age. AD is one of the leading causes of dementia and it is characterized by difficulty remembering recent events and showing signs of apathy and depression as early symptoms. Later signs include the impairment in communication, as well as confusion and difficulty in walking (2020 Alzheimer's Disease Facts and Figures, 2020). In short, pathologically speaking, the main indication of AD includes extracellular accumulation of Aβ plaque aggregates and intracellular formation of

neurofibrillary tangles (NFTs) made up of hyperphosphorylated tau proteins. These cellular changes ultimately lead to neuronal death and the progression of AD (Tiwari et al., 2019). The main focus will be on the pathology of AD characterized by Aβ accumulation. However, the pathology involving hyperphosphorylated tau will also be briefly discussed.

The pathogenesis of AD involving Aβ first involves the amyloid precursor protein (APP). A deviation from a normal, regulated cleavage of APP by β-secretase (BACE1) and γ-secretase results in the formation of insoluble Aβ fragments that cause neurotoxic effects. There are two main types of the Aβ fragments responsible for playing a role in neurotoxicity. First is Aβ40, which is more abundant but less neurotoxic than the second form, Aβ42, which is less abundant but more neurotoxic due to its higher insolubility. Nonetheless, the aggregation of these Aβ fragments results in altered neuronal function including blocking of ion channels, imbalance of calcium, increased oxidative stress, and reduced glucose and energy metabolism, resulting in cell death. The involvement of tau in AD pathogenesis is also due to the formation of the insoluble Aβ fragments. Normally, the tau protein works with tubulin to form stable microtubules. However, due to the presence of extracellular Aβ fragments, kinases become activated and the tau protein becomes hyperphosphorylated as a result. This causes instability in the microtubules, resulting in the collapse and conversion into tau filaments. These tau filaments accumulate to form NFTs, which are also highly insoluble and leads to an impairment in regular interneural communication and cell death (Tiwari et al., 2019).

Currently in this field, there is still extensive research going on in efforts to find viable therapeutic targets and methods to treat AD. A relatively new area of research looks into CatB, which is a lysosomal cysteine protease that may play an important role in the pathology of various neurological disorders. Previous studies have shown that Aβ results in lysosomal leakage which lead to cell death (Ditaranto et al., 2001; Ji et al., 2002). Furthermore, it was observed that the lysosomal leakage resulted in the release of CatB from the lysosome to the cytosol (Taneo et al., 2015). A schematic showing the possible involvement of CatB in the

pathology of AD and other related neurodegenerative diseases is shown in figure 1. These finding show the importance of CatB in the mechanism of the pathology of AD and other related neurological disorders and may be a potential drug target in treating these various neurological disorders.

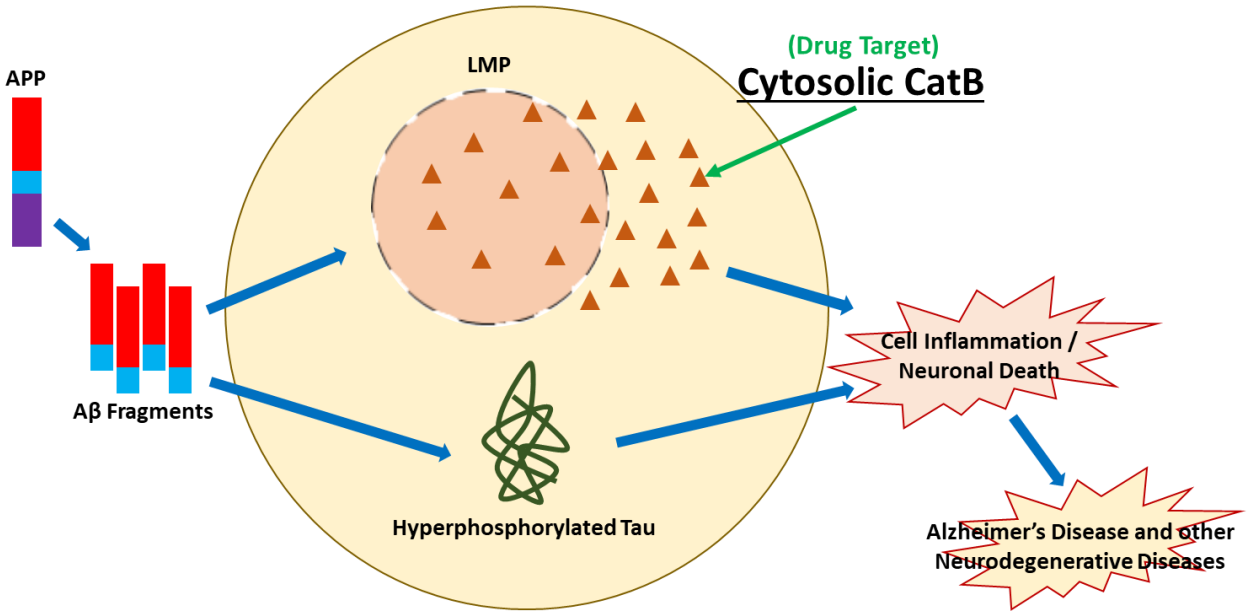


Figure 1. Hypothesis of cytosolic CatB involvement in AD and related neurodegenerative pathology.
The schematic above shows the critical mechanisms and the possible involvement of cytosolic cathepsin B (CatB) in the neuropathology of Alzheimer's disease (AD) and other related neurodegenerative diseases. The irregular cleavage of amyloid precursor proteins (APP) results in the accumulation of extracellular amyloid-beta (Aβ) fragments. These cause lysosomal membrane permeabilization (LMP) and release CatB into the cytosol, leading to cell inflammation and neuronal death and cause AD and other related neurodegenerative diseases. Aβ aggregates also result in hyperphosphorylated tau, which ultimately lead to AD and neurodegeneration. The link between lysosomal leakage of CatB and neurodegeneration makes CatB a promising therapeutic target for AD and other related neurodegenerative diseases.

C. Cathepsin B mechanism in Alzheimer's disease

1. Evidence for cathepsin B in cognitive deficits and neuropathology of Alzheimer's disease

CatB may be a worthwhile candidate to research as a therapeutic target for AD and other related neurodegenerative diseases. Table 1 shows evidence from previous findings that indicate CatB to be elevated in patients with AD, traumatic brain injury (TBI), and other patients with related dementia (Hook et al., 2020).

Table 1. Evidence of CatB elevation in patients of AD, TBI, and related dementia.
Table from Hook et al., showing evidence of elevated cathepsin B (CatB) in patients with Alzheimer's disease (AD), traumatic brain injury (TBI), and other forms of related dementia (2020).

Cathepsin B elevation in AD, TBI, and related dementia patients

Clinical Condition	Biofluid or Tissue	Cathepsin B Regulation	Features	Reference
Alzheimer's disease (AD)	plasma	↑	AD patients had 49% increase in CTSB protein above controls	(28)
AD	serum	↑	AD patients had ~45% increase in CTSB protein vs. controls ↑ CTSB correlated with MMSE * cognitive function scores indicating dementia	(29)
AD	plasma	↑	elevated CTSB protein in mild and severe AD by 50–80% above controls	(30)
AD	CSF	↑	CTSB protein was elevated by 1.9-fold in AD compared to control	(31)
AD	brain amyloid plaque	abnormal localization at amyloid plaques of AD brains	high CTSB activity and protein were abnormally localized in senile plaques of AD brains	(32)
Aging	CSF	↑	increased CTSB protein correlated positively with age	(35)
Severe Trauma	plasma	↑	CTSB activity was elevated 5–6 fold in severe trauma leading to organ failure	(36)
Multiple Trauma	plasma	↑	elevated CTSB associated with trauma and sepsis	(37)
Traumatic Brain Injury (TBI)	CSF	↑	elevated CTSB protein by 2-fold increase compared to controls	(38)
Inflammatory Neurological Diseases	CSF	↑	elevated CTSB activity in Guilllain-Barrer syndrome (GBS), chronic inflammatory demyelinating polyneuroopathy (CIDP), multiple sclerosis (MS), and meningitis	(39)

* Mini-mental state examination scores are utilized to monitor cognitive dysfunction, dementia (29).

There have also been several prior studies in mouse models that showed the involvement of CatB in the neuropathology of AD, as well as in the neurodegenerative phenotypes such as cognitive deficits. One study looked into the effects of CatB in transgenic mouse models of AD. It was found that by knocking out the gene for CatB, there was an improvement in the memory deficits in these mice. Furthermore, CatB gene deletion also resulted in the reduction of Aβ plaque loads in the brains of the transgenic mouse models (Kindy et al., 2012). Similarly, another study using mouse models exposed to *Porphyromonas gingivalis* lipopolysaccharide (PgLPS), which has previously been detected in AD patient brains, found that there was an improvement in learning and memory deficits with a deletion in the CatB gene. Moreover, the PgLPS-exposed mouse models with CatB knockout showed lower levels of CatB-dependent production of interleukin 1 beta (IL-1β), which is a proinflammatory cytokine

released in response to cell infection or injury (Wu et al., 2017; Lopez-Castejon and Brough, 2011). TBI, which results in brain dysfunction and pathology due to an external force, can be a valid general model for neurodegenerative diseases including AD due to the known increased risk for various age-related neurological diseases from repetitive or severe TBI. With this in mind, studies have shown that in TBI models, an overall improvement in behavioral deficits such as motor dysfunction was seen as a result of knocking out the gene for CatB (Hook et al., 2015). Figure 2 summarizes the mentioned previous studies that showed CatB gene knockout effects in AD and related disease models. These findings together show that CatB is involved in the neuropathology and cognitive deficits due to AD and related neurological diseases, thereby showing the potential of CatB as a therapeutic target.

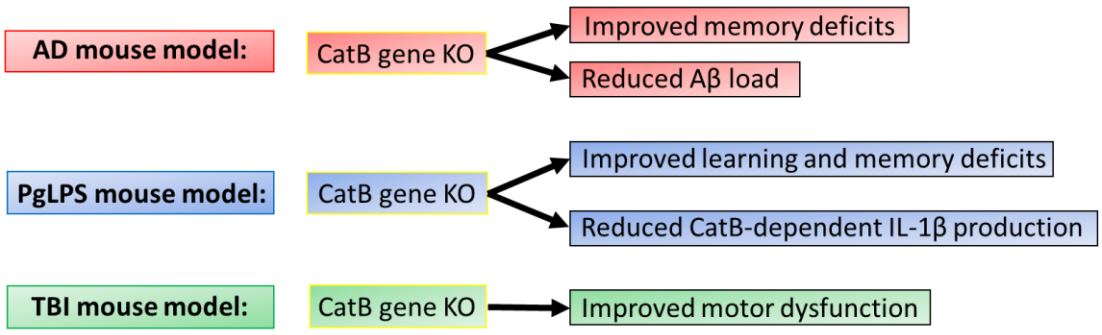


Figure 2. Evidence of CatB in neurodegenerative disease models of AD, PgLPS, and TBI. Effects of CatB gene knockout (KO) observed in AD, *Porphyromonas gingivalis* lipopolysaccharide (PgLPS)-exposed, and TBI mouse models. In a study by Kindy et al., CatB gene KO in AD mice showed improvement in memory deficits, as well as a reduction in amyloid-beta (Aβ) load in the brain (2012). In PgLPS-exposed mice, CatB gene KO resulted in learning and memory deficit improvement and a reduction in CatB-dependent production of interleukin 1 beta (IL-1β) (Wu et al., 2017; Lopez-Castejon and Brough, 2011). CatB gene KO in TBI mice in a study by Hook et al. showed motor dysfunction improvement (2015).

2. Cathepsin B and cathepsin lysosomal proteases

Lysosomal cathepsins are important proteases as they are required for the development and maturation of the brain by maintaining cellular protein homeostasis via protein degradation. They participate in various brain disorders that occur during different developmental age periods. Moreover, the dysregulation of cathepsins has been seen to occur in brain disorders including brain injuries, lysosomal storage diseases, and neurodegeneration. The cathepsin

protease family consists of cysteine cathepsins (B, C, F, H, K, L, O, S, V, W, and Z), aspartyl cathepsins (D and E), and serine cathepsins (A and G) (Hsu et al., 2018). CatB in particular is an important lysosomal protease due to its various roles in cell homeostasis.

CatB is a cysteine lysosomal protease classified as an enzyme belonging to the papain family. Its mRNA is originally translated as a preprocathepsin B enzyme in the rough endoplasmic reticulum, where it then gets transported and translated into a procathepsin B enzyme in the Golgi apparatus. With the removal and the final transport of the propeptide, the CatB enzyme is finally activated as mature CatB in the lysosome (Mort and Buttle, 1997). A unique characteristic of CatB is that it has been shown to have both endopeptidase and exopeptidase activities depending on the environment. At a low pH, as in the lysosomes, CatB has demonstrated to perform exopeptidase activities. However, in higher pH levels, it has been demonstrated to execute endopeptidase activities (Hook et al., 2015). It is known that out of all of the different lysosomal protease cathepsins, CatB is among one of the most abundant in the lysosome. The general physiological function of CatB is that it is involved in proteolysis (Yadati et al., 2020). One study has shown that CatB is involved in the cleavage of a specific calcium channel, MCOLN1/TRPML1 in the lysosome, affecting the calcium homeostasis. In addition, inhibition of CatB resulted in increased levels of gene encoding of TFEB, which is known to be a transcription factor important in controlling the expression of genes related to lysosomal autophagy. These finding demonstrate the importance of CatB in lysosomal biogenesis regulation as well as the regulation of autophagy (Man and Kanneganti, 2016). It has also been discovered that CatB is involved regulating hormones and growth factors for growth and development-related functions. The relevance of CatB in neuronal development is highlighted in a study that showed how mice models that lack both CatB and L resulted in the development of brain atrophy in the cerebral cortex as well as in the cerebellar region, leading to lethality in their early stages of life (Felbor et al., 2002). Together, CatB is an important protease shown to be

involved in many aspects of cell homeostasis and viability. Thus, it is an area of research worth looking into as a therapeutic target for AD and other related neurological diseases.

D. Cathepsin B and lysosomal leakage in Alzheimer's disease and neurodegenerative diseases

1. Evidence for lysosomal leakage in Alzheimer's disease and neurodegenerative diseases

Generally speaking, lysosomes are organelles within cells that are important for degrading and recycling extracellular and intracellular material in order to maintain cell homeostasis (Wang et al., 2018). In previous studies, lysosomal leakage, characterized as lysosomal membrane permeabilization has been found to be a part of the mechanism involved in the pathology of AD, as well as in other neurodegenerative diseases. Figure 3 summarizes these findings that will be mentioned more in detail in the next two paragraphs.

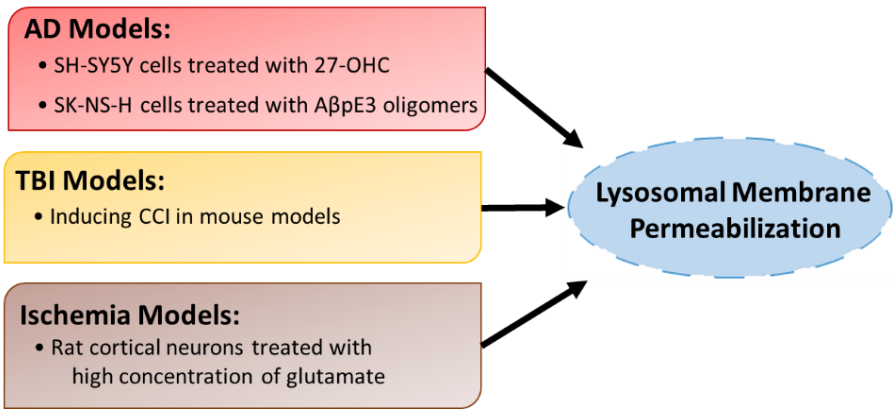


Figure 3. Evidence of LMP as the mechanism in the pathology of AD and other related neurodegenerative diseases.
A diagram summarizing the evidence of lysosomal membrane permeabilization (LMP) being a part of the mechanism in the neuropathology in models of Alzheimer's disease (AD), traumatic brain injury (TBI) and ischemia is shown. In AD models, a study by Chen et al. showed LMP and cathepsin B leakage as a result in SH-SY5Y cells when treated with 27-hydroxycholestrol (27-OHC) (2019). In another study, LMP was observed when $A\beta_{3(pE)-42}$ (AβpE3) oligomers were used to treat SK-NS-H cells (De Kimpe et al., 2013). LMP was observed when controlled cortical impact (CCI) was induced in TBI mouse models (Sarkar et al., 2020). In an ischemic model study by Yan et al., treating the rat cortical neurons with high concentration of glutamate resulted in LMP (2016).

In relation to AD, one study looked into lysosomal membrane permeabilization caused by cholesterol because of previous studies that linked cholesterol with neurodegeneration.

Specifically, 27-hydroxycholestrol (27-OHC) was utilized as increased levels of 27-OHC was seen in the serum and cerebrospinal fluid of patients with AD in several prior studies. Using the human neuroblastoma cell line, SH-SY5Y, and rat glial C6 cells, they found that treating them with 27-OHC resulted in lysosomal deficiency. This deficiency in lysosomes was discovered to be due to lysosomal membrane permeabilization. Another finding to mention in this study is that in the SH-SY5Y cells, treating them with 27-OHC resulted in the observation of CatB leakage into the cytoplasm around the lysosomes that underwent lysosomal membrane permeabilization (Chen et al., 2019). Another study also found lysosomal membrane permeabilization in AD, but instead using $A\beta_{3(pE)-42}$ (AβpE3), which is another variant of Aβ42 that was found to be present in AD and has also been suggested to be involved in the early stages of AD pathology. Using SK-NS-H human neuroblastoma cells, lysosomal membrane permeabilization was observed when the cells were treated with AβpE3 oligomers. They also found that AβpE3 treatment showed more extent of lysosomal leakage compared to Aβ42 (De Kimpe et al., 2013).

Other studies have looked into lysosomal leakage in other neurodegenerative diseases. One study looked into TBIs by using mouse models and inducing TBI via controlled cortical impact (CCI). By immunofluorescence staining of lysosomal membrane proteins and lysosomal enzymes, they determined there to be lysosomal membrane permeability when looking at brain sections from the mice after CCI induction (Sarkar et al., 2020). Another research studied lysosomal membrane permeabilization in regard to ischemic stroke. In such disease, as well as in other neurological diseases, glutamate has been seen to be involved in neuronal cell death because of excitotoxicity due to high glutamate concentration. By treating cultured cortical neurons from rats with high concentration of glutamate, they observed lysosomal membrane permeabilization. They also found that the lysosomal membrane permeabilization was mediated by reactive oxygen species (Yan et al., 2016).

These studies show the involvement of lysosomal membrane permeabilization in AD and other neurodegenerative diseases, therefore demonstrating the importance of lysosomal membrane integrity in maintaining neuronal homeostasis.

2. Lysosomal leakage signaling induces cell death and synaptic dysfunction: involvement of cathepsin B

Lysosomes are important for the overall homeostasis of cells. Figure 4 is a diagram that shows the different cell pathways that involves the lysosome.

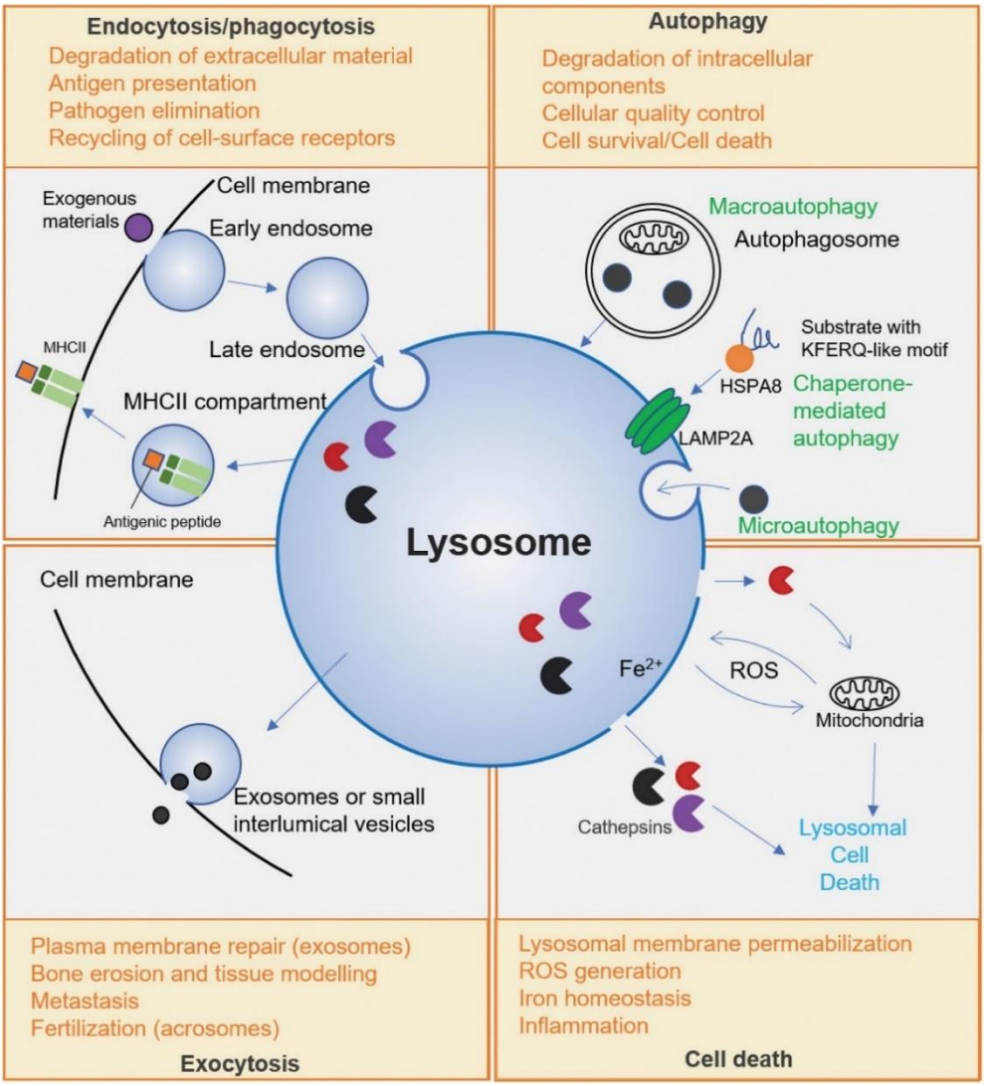


Figure 4. Lysosomal function in healthy and diseased cell.
A diagram from Wang et al., outlining the different cellular pathways that lysosomes are involved in in healthy and diseased cells (2018). Lysosomes are normally important in regulating cell viability via autophagy, endocytosis, and exocytosis functions. In diseased cells, a dysfunction in lysosomes, such as lysosomal membrane permeabilization is observed. This leads to cell/neuronal death, one of the causes being the release of cathepsins into the cytosol.

Many studies have shown that the dysfunction in the lysosomes, such as lysosomal membrane permeabilization or lysosomal leakage is seen in numerous diseases, including neurodegenerative disease (Wang et al., 2018). Several studies have found the implication of lysosomal membrane permeabilization in inducing cell inflammation and cell death. Using rat models for permanent middle cerebral artery occlusion (pMCAO), which is a model for cerebral ischemia, the study has found that in the ischemic astrocytes, by using CA-074Me, an inhibitor of CatB, it prevented pMCAO-induced lysosomal release of CatB into the cytosol. They also observed that it prevented caspase-3 activation, which is part of the cell apoptotic pathway (Xu et al., 2014). Another study looked into cells of glioblastoma, which is a form of cancer in the central nervous system. They found that by using Thymoquinone (TQ), which is a compound previously seen to induce apoptosis in other cells, there was an induction of lysosomal membrane permeabilization, which resulted in lysosomal leakage of CatB into the cytosol. This mediated cell death, but was prevented when a CatB inhibitor, Cathepsin inhibitor III, was used (Racoma et al., 2013).

Lysosomes are also important in maintaining proper synapse function, as a proper functioning autophagy system which lysosomes are an essential aspect of, is necessary in the central nervous system to prevent protein aggregate accumulation and neurodegeneration due to synapse dysfunction (Fassio et al., 2019). Using MPS-IIIA mice models, which is representative of lysosome storage disorders, a study has showed that in the hippocampal neurons in these mice, lysosomal enlargement and lysosomal dysfunction was observed, resulting in a reduction in the rate of exocytosis in the presynaptic terminal. Furthermore, there was a loss in the SNARE complex proteins, SNAP25 and VAMP2, which are important in regulating synaptic vesicle trafficking in the presynaptic terminal. However, restoration of lysosome activity showed an increase in the SNAP25 and VAMP2 protein levels (Sambri et al., 2016). Moreover, deletion of presenilin, which is normally important in lysosomal proteolytic clearance, has shown to disrupt vATPase proton pumps, which are important in maintaining

lysosomal pH. vATPase disruption results in deacidification of lysosomes, which leads to substrate accumulation. This is associated with tau hyperphosphorylation as seen in AD (Nixon, 2017).

These findings outline the importance of lysosomes in maintaining cell homeostasis. A dysfunction, such as lysosomal leakage is evident in the involvement of cell inflammatory response and cell death, as well as synaptic dysfunction, which are all aspects implicated in neurodegenerative diseases.

3. Involvement of microglial-mediated inflammation and cathepsin B participation in cognitive deficits and neurodegeneration

Microglia are an important set of immune cells in the brain, making up anywhere from 5 to 12 percent of cells in the brain. They function to maintain homeostasis in the brain and defend the brain against any type of pathogens and neurodegenerative diseases including AD (Hickman et al., 2018). Though its main function is to protect the brain, several studies have shown the involvement of microglial CatB in neurodegeneration. Lowry and Klegeris summarized how the activation of microglia as a result of pro-inflammatory stimuli eventually leads to the upregulation of CatB. This causes downstream effects that ultimately leads to the activation of IL-1β, as well as neurotoxicity (2018). A diagram summarizing this mechanism is shown in figure 5a.

Regarding AD, in microglial BV2 cells, a study by Gan et al. found that activation of microglia by Aβ42 resulted in cell death. They also identified the genes that were upregulated by Aβ42 and found that the gene for CatB was among one of them. Further analysis was then performed regarding CatB where they used small interfering RNAs to specifically silence CatB gene expression. This showed complete eradication of the neurotoxic effects previously seen in the Aβ42-activated microglia. Moreover, they used a CatB inhibitor, CA-074 which also showed improvements, coming to the conclusion that CatB is involved in the neurotoxicity (2004).

Another study utilized chromogranin A (CGA), which is a peptide known to be upregulated in AD and other neurodegenerative diseases, to study the effects on microglia. They found that CatB was secreted in CGA-treated microglia. Furthermore, using the media that resulted from activated microglia, HT22 hippocampal cells that were treated with the medium showed significant cell death. However, when a CatB inhibitor, z-FA-fmk, or a CatB antibody was used, the neuronal death from the CGA-activated microglia media was reduced. Similar results were seen in cerebellar granule cells using the CatB antibody. These results show the involvement of microglial CatB in neuronal death (Kingham and Pockock, 2001).

There was also another study looked into microglial CatB in regard to Niemann-Pick disease type A (NPA), which is a lysosomal storage disorder characterized by neurodegeneration, demyelination, and the accumulation of lysosomal lipids. This disease is known to be caused by a loss of function mutation in the gene for acid sphingomyelinase (ASM). The study established that mice models with the deletion of the ASM gene result in amoeboid microglia in the areas prone to neurodegeneration, similar to that of NPA patients. Therefore ASM-knockout (ASMko) mouse models were used for their experiments. In the ASMko microglia, lysosomal leakage was observed, accompanied with the extracellular release of CatB into the media. Moreover, when hippocampal neurons from the wildtype mice was treated with the media of the ASMko microglia, neuronal death was observed, demonstrating the involvement of cathepsin B in neuronal death. They further used a cathepsin B inhibitor, Ca074Me in ASMko mice to observe effects on NPA phenotype *in vivo* and found that inhibiting cathepsin B resulted in no NPA phenotype based on motor performance analysis (Gabande-Rodriguez et al., 2018).

Together, these studies demonstrate the importance of microglial CatB studies, as they show how microglial CatB takes part in neurodegeneration, as well as cognitive impairment in neurodegenerative disease. A flowchart summarizing the general involvement of microglial CatB

in various neurodegenerative models from prior studies that were mentioned above is shown in Figure 5b.

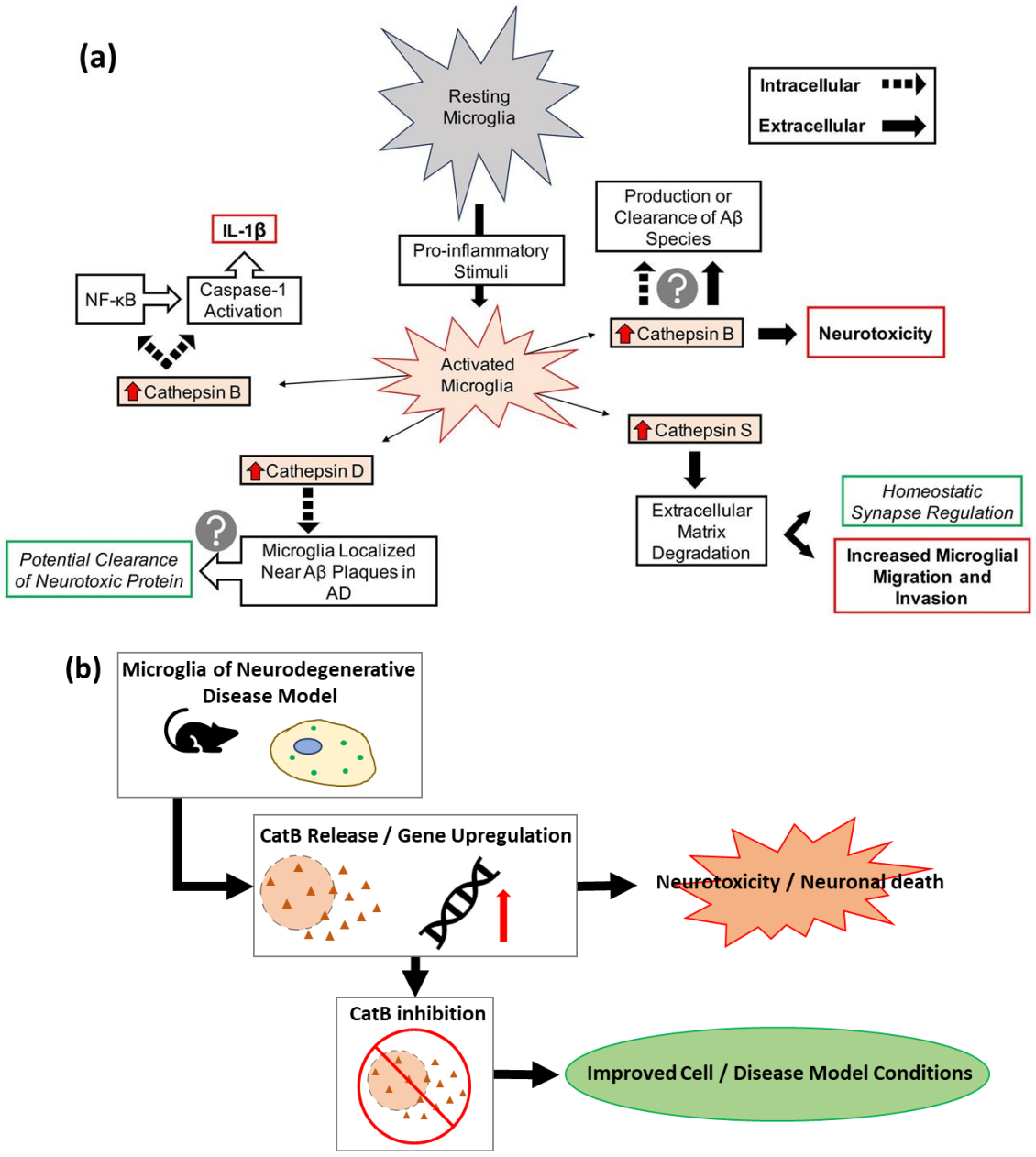


Figure 5. Inflammatory and neurotoxic effects of CatB in activated microglia and evidence of microglial CatB activity in AD and related neurodegenerative disease models.
(a) Diagram from Lowry and Klegeris showing how activation of microglia due to inflammatory stimuli leads to cathepsin B (CatB) upregulation. This results in interleukin 1 beta (IL-1β) activation showing cell inflammation, and also neurotoxicity (2018). (b) Based on several prior studies done on microglia of neurodegenerative disease models including Alzheimer's disease (AD), microglial activation has been shown to induce CatB release and the upregulation of the CatB gene, ultimately leading to neurotoxicity and cell death. Using different CatB inhibitors improved the conditions of the cell and neurodegenerative disease animal models (Gan et al., 2004; Kingham and Pockock, 2001; Gabande-Rodriguez et al., 2018).

4. Lysosomal leakage of cathepsin B in microglia leads to cognitive deficits in aging and periodontitis-associated Alzheimer's disease

The link between lysosomal leakage of CatB and microglia, and its implications in cognitive deficits in aging and periodontitis-associated AD is demonstrated based on two separate studies. The first study, as briefly mentioned previously in section C1, is the study by Wu et al. where they used PgLPS-exposed mouse models to demonstrate that there was an improvement in learning and memory deficits with the deletion of the CatB gene. They also showed lower levels of CatB-dependent production of IL-1β. Going more into detail, the main focus of the study was to look into the association between periodontitis, which is a common chronic oral bacterial infection, and the cognitive decline in AD, as various clinical and experimental studies have shown strong association between periodontitis and cognitive decline in AD. The study also looked into the effects of PgLPS on microglia and found that PgLPS induced the activation of microglia in a CatB-dependent manner. They also found that this activation of microglia was required for the intracellular accumulation of Aβ in primary cultured hippocampal neurons of wild-type mice (2017).

For the second study, it is considered that oxidative stress and chronic neuroinflammation, which can occur from regular aging, cause motor and cognitive function deficits, with microglial activation being the main source for the oxidative stressors and proinflammatory mediators in the brain. With evidence from prior research, such as the previously mentioned first study, CatB is a candidate for this study to look into how the oxidative stress and neuroinflammation may arise during aging. They found that, in the hippocampus of CatB-deficient mouse models, there was an improvement in the age-dependent oxidation and inflammation. The CatB-deficient mice also showed improvement in the age-dependent decline of learning and memory. Further studies using microglia showed that mimicking aged microglia by causing lysosomal disruption via treatment with a lysosome-destabilizing agent, LLOMe,

results in the leaking of CatB to the cytosol, thereby increasing the generation of oxidation stressors and proinflammatory mediators (Ni et al., 2019).

These findings together show the evidence of oxidative stressors and neuroinflammation due to lysosomal leakage of CatB in microglia, which ultimately leads to cognitive deficits seen in aging and periodontitis-associated AD.

E. Research: Investigation of Aβ42 induction of cell death and lysosomal leakage

1. Specific Aims

Aβ oligomers are neurotoxic peptides found to accumulate in brains of AD, one of these forms being Aβ42. In AD and other neurodegenerative diseases in general, neuronal cell death is the ultimate end point within the neuropathology (Tiwari et al., 2019). One of the mechanisms involved in neuronal cell death is lysosomal leakage as previous studies have shown that Aβ42 induces lysosomal leakage (Ji et al., 2002). Lysosomal leakage also results in the release of lysosomal proteases into the cytosol. One particular group of lysosomal proteases that is of interest are cysteine cathepsins, specifically CatB, as there is evidence from previous research of its involvement in the neuropathology of AD and other neurodegenerative diseases (Taneo et al., 2015; Kindy et al., 2012).

In this project the association of cell death due to Aβ42-induced lysosomal leakage is investigated using human neuroblastoma cell cultures, SK-N-MC and SH-SY5Y, and BV2 microglial cell cultures. The question addressed by this research is: do Aβ42 oligomers induce lysosomal leakage of CatB concomitantly with Aβ42 oligomer-induced cell death? While Aβ42 oligomers are known to induce lysosomal leakage, induction of CatB translocation from lysosomes to the cytosol has not yet been demonstrated. Therefore, the specific aims of the study are: (1) By treating neuroblastoma cell cultures with Aβ42 oligomers, the extent of cell death is monitored via a lactate dehydrogenase (LDH) assay. (2) Then, the degree of lysosomal leakage induced by Aβ42 oligomers is measured by fluorescent imaging using acridine orange

(AO) staining. (3) In BV2 microglial cells, sucrose density gradient centrifugation is used to isolate cytosolic fractions and the presence of CatB is analyzed via western blot. (4) Finally, in SK-N-MC and SH-SY5Y cells, using a bifunctional probe, cysteine cathepsin activity is measured in a cytosolic, neutral pH environment.

2. Methods

Human neuroblastoma and BV2 microglial cell cultures

For SK-N-MC and SH-SY5Y human neuroblastoma cell cultures, as well as the BV2 microglial cell cultures, the same protocol was utilized, with the only difference being the complete media used. For both neuroblastoma cell cultures, MEMα with 10% FBS is used, and for BV2 microglial cell cultures, RPMI with 10% FBS is used. First, 10 mL of complete media was pipetted into a T25 flask. The flask was then put into the incubator for at least 15 minutes to equilibrate with 5% CO_2 at incubator temperature of 37 °C. Next, frozen cryotube of cells from liquid nitrogen storage is obtained. Thaw the cells until barely thawed by placing the bottom of the tube in a 37°C water bath for about 1 minute. After, immediately rise tube with 70% ethanol, dry with kimwipe and put in the hood. Transfer the cells to 10 mL complete media in a 15 mL sterile tube and mix gently by trituration. Then, transfer the cells into a T25 flask and place in the incubator. Check the cells daily to monitor progress: if they are not growing well by the third day, try a new cryotube. When the cells are ready to split, with around 70-90% confluency, passage and transfer to 2-3 T75 flasks. Grow the cells to 70-90% confluency and freeze down the replacement frozen stocks.

Aβ42 oligomer preparation and treatment

For the preparation of Aβ42 oligomers, allow the 1 mg vial of Aβ42 peptide to equilibrate at room temperature in the manufacturer's vial for 30 minutes to avoid condensation upon opening the vial. In the fume hood, resuspend the peptide in 221 µL of ice-cold HFIP to obtain a

1 mM solution. Vortex for a few seconds and quickly after, using a 100 µL capacity Gas Tight Hamilton Syringe, divide equally into 4 tubes (55 µL/tube in 2 mL tubes) so there is 0.25 mg per tube and seal the tubes with parafilm. Next, incubate for 2 hours at room temperature to allow monomerization. After, open the vials and concentrate under vacuum using the SpeedVac centrifuge (800g at room temperature for about 15 minutes) until completely dried down and a film is observed at the bottom of the vials. Make sure to monitor the temperature of the centrifuge at 25 °C to avoid peptide degradation. Then, add 22 µL of DMSO to each film and sonicate the 4 tubes in a water bath for 10 minutes to ensure complete resuspension. After, add 550 µL of MEMα (for SK-N-MC and SH-SY5Y neuroblastoma cells) or RPMI (for BV2 microglial cells) to each tube (100 µM concentration) and store in the refrigerator overnight. The next day, vortex each Eppendorf tube for 30 seconds and dilute 1:4 in serum-free MEMα or RPMI media for a 25 µM final concentration.

For the treatment of the cells with Aβ42 oligomers, obtain the prepared 25 µM Aβ42 oligomer sample, as well as a 25 µM sample of DMSO in the appropriate media (Memα for SK-N-MC and SH-SY5Y neuroblastoma; RPMI for BV2 microglia) to be used as the DMSO control. Remove the growth media from the cells and wash the flasks twice using 10 mL of PBS per wash. Then, add 8.8 mL of the 25 µM Aβ42 oligomer or the 25 µM DMSO control sample to the cells and return the cells to the incubator at 37 °C with 5% CO_2.

Cell death monitored by LDH in the media

LDH assay is utilized to monitor cell death of the neuroblastoma cell samples. Figure 6 shows the mechanism in which the LDH assay works that allows for the monitoring of cell death. When the cell membrane is damaged, LDH (a cytosolic enzyme present in many cell types) gets released into the media. This extracellular LDH is quantified via a coupled enzymatic reaction where LDH catalyzes the conversion of pyruvate from lactate through NAD+ reduction to NADH. Diaphorase, through NADH, is able to reduce tetrazolium salt (INT) to red formazan, which can

be measured at 490nm. Because formazan formation is directly proportional to extracellular LDH, the level of formazan, therefore, the level of NADH, is indicative of cytotoxicity.

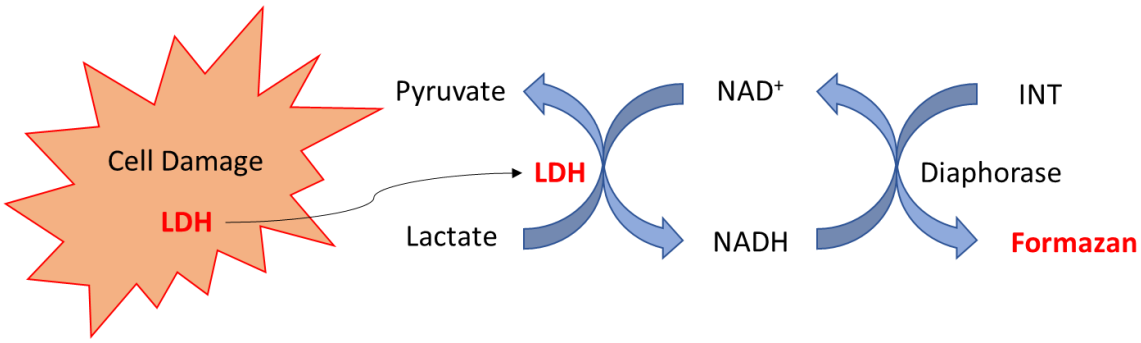


Figure 6. LDH Assay Mechanism.
The diagram shows the overall mechanism of how the lactate dehydrogenase (LDH) assay is utilized to determine the extent of cell death. When there is cell death due to plasma membrane damage, LDH is released into the media of the cell culture. As a result, LDH catalyzes the reaction in which lactate is converted to pyruvate through the reduction of NAD+ to NADH. Then, NADH is utilized by diaphorase to reduce tetrazolium salt (INT) to formazan, which is a red compound that is measured at 490 nm. Thus, the red fluorescence from formazan indicates cell death because the level of formazan is directly proportional to the amount of LDH that is released into the media.

The protocol utilized for the LDH assay is as follows: first, the reaction mixture is prepared. Dissolve 1 vial of the substate mix (lyophilizate) with 11.4 mL of water in a 15 mL conical tube and gently mixing until fully dissolved. Next, thaw 1 vial of the assay buffer (0.6 mL) at room temperature – make sure to protect from light and to not leave at room temperature longer than necessary. Finally, the reaction mixture is prepared by combining the 0.6 mL assay buffer with 11.4 mL of substrate mix in a 15 mL conical tube. Mix well and protect from light until use. The LDH assay is then performed: a summary protocol is shown is shown in figure 7. First, in a 96-well plate, add 50 µL of NADH standard, supernatant samples from treated cells, or buffer blanks. Second, add 50 µL of the reaction mixture that was prepared previously to each well and incubate for 30 minutes at room temperature. After incubation, add 50 µL of stop solution to each well. Lastly, use a spectrophotometer to measure the absorbance at 490 nm and 690 nm to determine the LDH activity.

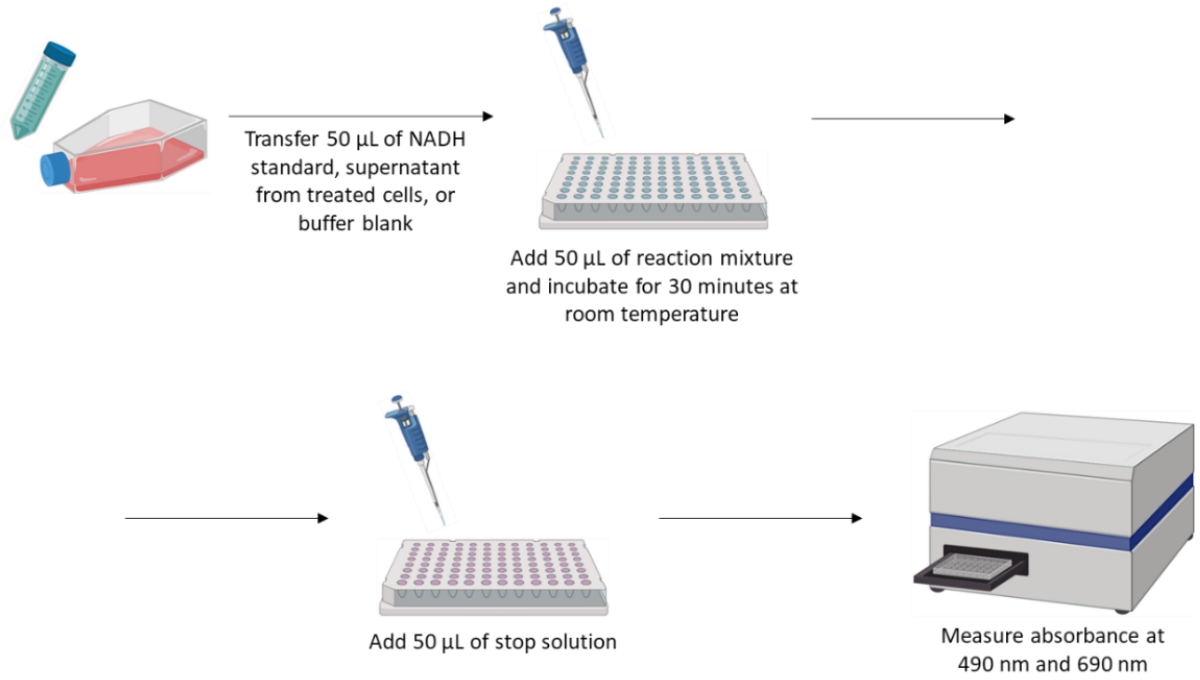


Figure 7. LDH Assay Protocol.
The diagram shows the steps involved in the lactate dehydrogenase (LDH) assay. 50 µL of the NADH standard, supernatant from treated cells, or buffer blank are transferred to a 96 well plate. Then 50 µL of the reaction mixture is added and incubated for 30 minutes at room temperature. After, 50 µL of the stop solution is added and the absorbance is measured at 490 and 690 nm using a fluorescent plate reader.

Lysosomal leakage measured by AO

To measure lysosomal leakage, AO is utilized. AO is a fluorescent dye that is able to cross the cell membrane easily. Due to its properties as a weak base, it can accumulate in lysosomes which have a low internal pH. Outside of lysosomes, acridine orange binds to cellular DNA as a monomer. This results in a green fluorescence when excited with blue light. However, acridine orange binds to lysosomes as polymers, resulting in a red fluorescence upon blue light excitation. The dye is protonated once it is inside the lysosomes so it will stay inside as long as the lysosome is intact (Vermes and Hannen, 1994; Grunwald, 1993).

For the experimental protocol, first, a 1mg/mL stock solution of AO is made by dissolving in sterile in ddH_2O. Then, using the SK-N-MC and Sh-SY5Y that is grown to about 50% confluency, aspirate old media and replace with PBS containing 5 µg/mL of AO (1:200 dilution of stock in media, which is 50 µL of 1 mg/mL AO stock in 10 mL media) and incubate for 5 minutes at 37 °C. After, fluorescent images are acquired using ImageXpress Micro NTS

automated plate reader (company: Molecular Devices) at 20X magnification – four 20X fields are acquired per well. The excitation peak for green and red fluorescence are 502 nm and 460 nm, respectively. The emission peaks are 525 nm for green and 650 nm for red. After obtaining the fluorescent images, they are analyzed using MetaXpress software (Molecular Devices) to determine the fluorescence intensity of red and green channels. Red fluorescence is set to determine the intensity of subcellular spot features within each cell and the green fluorescence is set to determine the intensity if cytoplasmic area of each cell. Finally, calculate the fluorescence per cell using the equation: $\frac{(Red\ spot\ intensity) \times (\#\ of\ spots)}{(Average\ green\ intensity)}$.

Aβ42-induced translocation of lysosomal cathepsin B into the cytosol assessed by western blot

The cytosolic fraction samples of BV2 microglial cells for the western blot analysis was obtained through the sucrose density gradient centrifugation method. Sucrose density gradient centrifugation utilizes different density gradients of sucrose to allow for the separation of the sample based on their density. First, the solutions for fractionation are prepared, which are to be kept at 4 °C. Initially, 50 mL of 2.2 M sucrose is made by adding 37.6 g of sucrose to 25 mL of water, allowing it to dissolve on a stir plate for about 20 minutes, pouring into a graduate cylinder, and bringing the volume up to 50 mL by adding water. Then, 30 mL of 0.32 M sucrose is made by adding 25.64 mL of water to 4.36 mL of the 2.2 M sucrose solution. Next, 10 mL of 0.8 M sucrose solution is prepared by adding 6.37 mL of water to 3.63 mL of the 2.2 M sucrose solution. Finally, 10 mL of 1.5 M sucrose is made by adding 3.18 mL of water to 6.82 mL of the 2.2 M sucrose solution. After the solutions of sucrose at four different concentrations are prepared, harvest the BV2 microglial cells and suspend with 0.6 mL of 0.32 M sucrose and transfer to a 1 mL Wheaton tissue grinder that is placed in an ice bucket. Then, homogenize with tight pestle (clearance of 50 μm) with 10 strokes on ice and transfer to Thermo 10 mL tubes with screw caps. Balance the tubes to within 0.1 g of each other using 0.32 M sucrose. First, a

low-speed differential centrifugation is performed, followed by ultracentrifugation to isolate the cytosol. Figure 8 shows a summarized diagram for both of these centrifugation methods. For the low-speed centrifugation method, centrifuge the previously prepared tubes at 600 x g for 10 minutes. Transfer supernatant (S1) to a new 10 mL screw cap tube. Then, centrifuge S1 at 800 x g for 15 minutes. Collect the supernatant (S2) for ultracentrifugation by transferring S2 into chilled 1.5 mL Eppendorf tube. For the ultracentrifugation method, prepare gradients as shown figure 8b: 0.5 mL of 2.2 M sucrose at the bottom, followed by 2 mL of 1.5 M sucrose, and then 2 mL of 0.8 M sucrose. Layer 0.5 mL of the S2 from last low-speed centrifugation method on top of the 0.8 M sucrose layer. Place weight-balance tubes in the SW-60 rotor using 0.32 M sucrose to bring all tubes within 0.01 g of each other. Release the vacuum on the ultracentrifuge, open the door, place the tubes in the rotors, and close the door. Set centrifuge speed to 100,000 x g and start centrifuging for 90 minutes. While centrifuging, prepare Eppendorf tubes to collect the fractions by pre-chilling in the refrigerator. After ultracentrifugation has finished, remove buckets from the rotor and place on ice. After, carefully lift out the tubes and place on the ice. Finally, collect 150 µL of fraction samples starting at the top of the tube and store the fractions at -70 °C. The cytosolic fractions will be used for the anti-cathepsin B western blot.

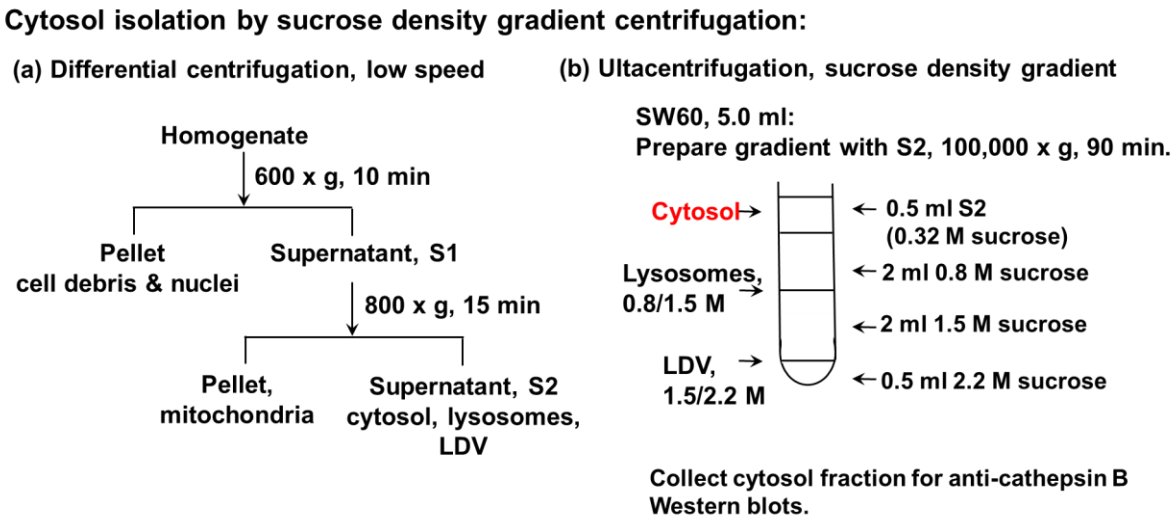


Figure 8. Low-speed differential centrifugation and ultracentrifugation methods for cytosol isolation by sucrose density gradient centrifugation.
The diagrams above show the summary of the methods involved in the sucrose density gradient centrifugation protocol. (a) Shows the low speed, differential centrifugation method where the cell homogenate is first centrifuged at 600 x g for 10 minutes, separating into supernatant S1, and pellets containing cell debris and nuclei. Then supernatant S1 is centrifuged at 800 x g for 15 minutes, separating into supernatant S2, which contains the cytosol, lysosomes, and large dense core vesicles (LDV), and pellets containing mitochondria. (b) Shows the ultracentrifugation method where supernatant S2 is layered on top of the different sucrose density gradients as shown in the diagram. The contents of S2 are separated by centrifuging at 100,000 x g for 90 minutes. This allows the isolation of the cytosolic fraction, as it resides in the top layer in 0.32 M sucrose, which is extracted and used for the anti-cathepsin B western blot.

For the western blot, obtain a 4X lithium dodecyl sulfate (LDS) sample buffer and bringing it to room temperature, making sure the contents are dissolved. Then, the following sample are prepared in Eppendorf tubes: 1X LDS sample buffer, 50 mM of Dithiothreitol (DTT), and the cytosolic fraction sample. Vortex the samples and heat the sample at 95 °C in a heating block for 10 minutes. While heating, prepare 600 mL of 1X Invitrogen running buffer (20X stock). Then, obtain gel, open the sealed gel, and remove the comb and tape. Wash the wells with 1X running buffer using a transfer pipet and assemble the gel apparatus in a gel box. After, load the samples in the lanes while leaving the first and last wells empty. Run the gel at 60 V until the samples migrate through the stacking gel and then increase the voltage to 120 V. When the dye front approaches the bottom of the gel, turn the power supply off and remove the gel. Next, open the gel casing with a gel spatula and prepare 300 mL of 1X Invitrogen transfer buffer (20X

stock) with 10% methanol. For the transfer of the gel, soak 4 sponges, 4 pieces of filter paper, and 1 piece of nitrocellulose membrane cut to 8 cm x 9 cm. Assemble the transfer 'sandwich' starting at the bottom (cathode side) with 2 sponges, followed by 2 filter papers, gel, nitrocellulose membrane, 2 filter papers, and finally, 2 sponges at the top (anode side). Make sure to have no bubble between the gel and the nitrocellulose membrane. Then, place the transfer 'sandwich' in the transfer chamber and place in the transfer box. The current should run from the gel to the membrane for the transfer. Next, run the transfer at 30 V for 1 hour. After the transfer, place the nitrocellulose membrane in blocking solution (5% dry milk in tris-buffered saline (TBST)) and rock gently for 1 hour. After, rinse with TBST. Then, incubate in mouse CatB antibody (polyclonal goat IgG, R&D systems AF965) on a ricker in a cold room overnight. Next day, rinse the membrane three times with TBST and fill with fresh TBST. Place on rocker for 15 minutes. Rinse and rock the membrane three times for 5 minutes each. After rinsing, incubate the membrane in secondary antibody for 1 hour at room temperature on a rocker. After 1 hour, obtain Femto Western detection reagent kept at 4 °C and bring to room temperature. Wash the secondary antibody-incubated membrane by repeating the rinse and rock step previously done with the primary antibody-incubated membrane. Remove TBST from the membrane completely and incubate in 5 mL of detection reagent on a rocker for at least 5 minutes. Finally, using a ChemiDoc imager, obtain the western blot image.

Cytosolic neutral pH activity of cysteine cathepsins measured by bifunctional probe

A bifunctional probe was used to measure cysteine cathepsin activity at a cytosolic neutral pH. A diagram illustrating the general mechanism of a bifunctional probe is shown in figure 9. In a bifunctional probe, it contains 2 fluorescent reporter elements (fluorophores). The purpose of one (activity-dependent) fluorophore is to become fluorescent upon binding of an active protease, such as a cathepsin, to the probe and the other (pH-dependent) fluorophore is to become fluorescent and change in gradient depending on the pH. Specifically for this

research, a pH-dependent fluorophore that show maximal fluorescence at neutral pH is used to look at cathepsin activity in non-acidic areas. The activity dependent fluorophore used is sulfo-Cy5 which was previously validated for cathepsin use when paired with a sulfo-QSY21 quencher. Cy5 is an ideal choice as it has low fluorescent background and is more binding specific than other hydrophobic dyes. QSY21 is an ideal choice as it absorbs fluorescence from 600 to 700 nm which is the entire emission spectrum of Cy5. For the pH-dependent fluorophore, Oregon Green 488 (OG488) is used as it is spectrally distinct from Cy5, which prevents fluorescent resonance energy transfer and allowing for independent measurement of protease activity and pH. The OG488 fluorophore is specifically used in the LES12 bifunctional probe, which is what was utilized for this project. Initially, when the probe is not bound to anything, there is no fluorescence. As it binds to a cysteine protease, there will be a red fluorescence indicating protease (cathepsin) activity, and the probe will have a green fluorescence when it is at a neutral pH. Upon obtaining the two separate images with different fluorescence, merging the imaging may reveal yellow fluorescence due to co-localization of the activity- and pH-dependent fluorophores. This will indicate the protease activity occurring at neutral pH (Sanman et al., 2016).

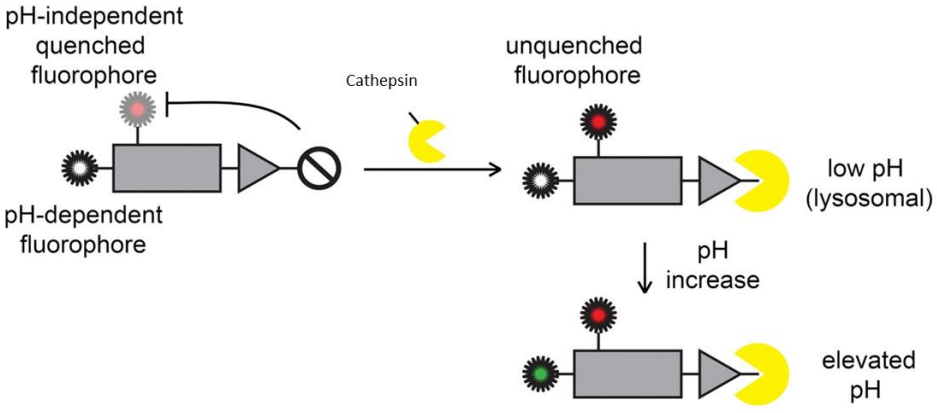


Figure 9. Bifunctional Probe Mechanism.
The diagram shows the mechanism of a bifunctional probe, obtained from Sanman et al. (2016). A pH-independent fluorophore and a pH-dependent fluorophore is contained in the probe. Initially, when the probe is not bound, there is no fluorescence. However, when it binds to a cysteine protease, a red fluorescence is observed, indicating protease activity. When the pH of the probe environment is increased to around a neutral pH, there will also be a green fluorescence. Fluorescence of both red and green, resulting in a yellow fluorescence due to co-localization, would indicate protease activity occurring at a neutral pH.

As for the protocol of the experiment, the 20 mM LES12 bifunctional probe (Cy5 (red)-tagged activity probe + low pH OG488 (green) tag at 4.8 pKa) is obtained in DMSO. First, incubate the live cells with 5 μM probe in 100 μL media per well: with the LES12 probe, add 0.3 μL of the 20 mM stock to 1.2 mL of MEMα for SK-N-MC cells, and add 0.3 μL of the 20 mM stock to 1.2 mL of MEMα/F12K for SH-SY5Y cells. Then, remove the media and replace with serum-free media containing the probe and incubate the probe for 2 hours at 37 °C. After incubation, wash with DPBS three times, then change to serum-free media without the probe (MEMα for SK-N-MC cells and MEMα/F12K for SH-SY5Y cells). Next, obtain two 20X images per well using ImageXpress Micro (company: Molecular Devices)The excitation peaks for Cy5 (red) and OG488 (green) are 420 nm and 650 nm, respectively. The emission peak for Cy5 is 520 nm and 670 nm for OG488. After obtaining the images, they are analyzed using MetaXpress software (Molecular Devices) to determine the average red and average green fluorescence intensity per field and calculate using the equation: $\frac{(Average\ red\ intensity\ per\ field)}{(Average\ green\ intensity\ per\ field)}$.

3. Results

Aβ42 oligomers induce cell death in neuroblastoma cells

The results for the LDH assay for SK-N-MC neuroblastoma cells is shown in figure 10. When SK-N-MC cells were treated with Aβ42 oligomers, based on the LDH assay, there was a significant increase of about 50% in the amount of NADH compared to the DMSO control 24 hours after treatment and about a two-fold increase 48 hours after treatment. With the H_2O_2 treatment, there was a slight significant increase in NADH levels compared to the water control 24 hours after treatment. The results for the LDH assay for SH-SY5Y neuroblastoma cells is shown in figure 11. When SH-SY5Y cells were treated with Aβ42 oligomers, there was a significant increase of about 50% in NADH 48 hours after treatment. When treated with H_2O_2, there was a slight significant decrease in NADH levels compared to the control 24 hours after treatment.

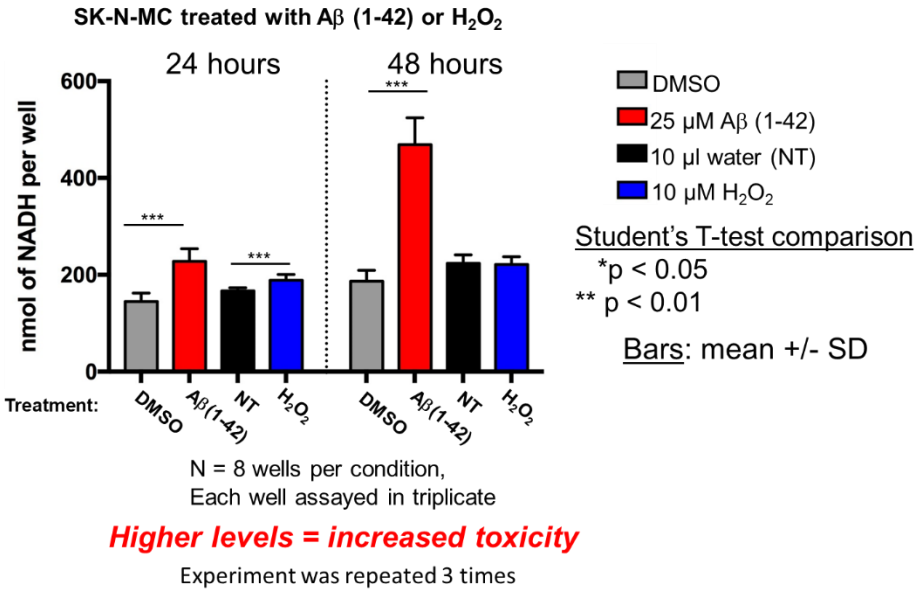


Figure 10. LDH assay result of SK-N-MC cells treated with Aβ42 oligomers.
The bar graph shows the average amount of NADH (nmol) measured per well from the LDH assay for SK-N-MC neuroblastoma cells treated with Aβ42 oligomers or H_2O_2 compared to their respective controls of DMSO or water (NT), 24 and 48 hours after treatment. Higher levels of NADH indicate increased cell toxicity. Treatment with Aβ42 oligomers showed a significant increase of about 50% after 24 hours and about a two-fold increase 48 hours after treatment compared to the DMSO control. Treatment with H_2O_2 showed a slight significant increase 24 hours after treatment compared to water control.

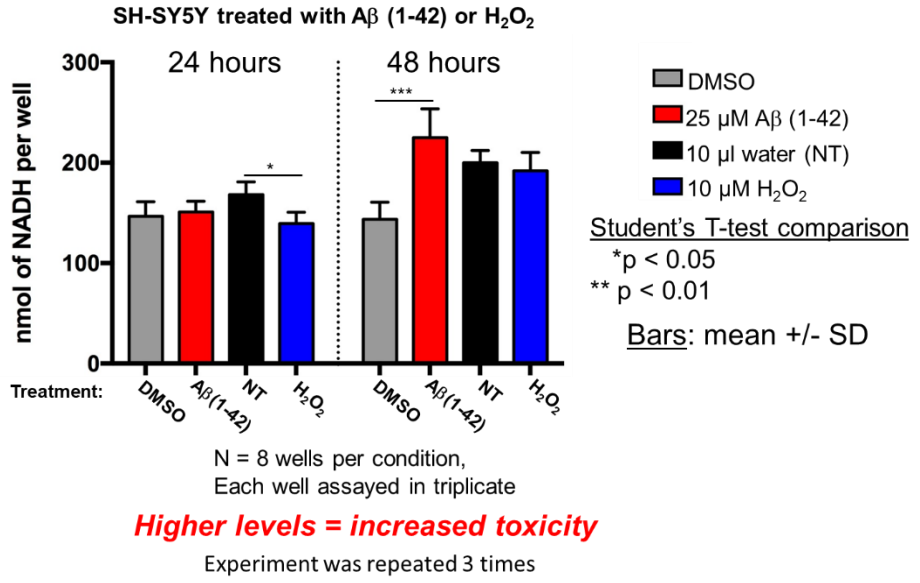


Figure 11. LDH assay result of SH-SY5Y cells treated with Aβ42 oligomers.
The bar graph shows the average amount of NADH (nmol) measured per well from the LDH assay for SH-SY5Y neuroblastoma cells treated with Aβ42 oligomers or H_2O_2 compared to their respective controls of DMSO or water (NT), 24 and 48 hours after treatment. Higher levels of NADH indicate increased cell toxicity. Treatment with Aβ42 oligomers showed a significant increase of about 50% 48 hours after treatment compared to the DMSO control. Treatment with H_2O_2 showed a slight significant decrease 24 hours after treatment compared to water control.

Aβ42 oligomers stimulate lysosomal leakage

An example fluorescent imaging from acridine orange staining of SK-N-MC neuroblastoma cells is shown in figure 12. The results from the acridine orange staining for SK-N-MC neuroblastoma cells is shown in figure 13. In SK-N-MC cells, acridine orange staining showed that, when treated with Aβ42 oligomers, there was about a significant five-fold decrease in the red fluorescence intensity compared to the DMSO control 24 hours after treatment. Treatment with H_2O_2 showed about a three-fold decrease compared to the no treatment control 24 hours after treatment. The results from the acridine orange staining for SH-SY5Y neuroblastoma cells is shown in figure 14. With SH-SY5Y cells, acridine orange staining showed a about a significant five-fold decrease in intensity compared to the DMSO control 48 hours after Aβ42 treatment.

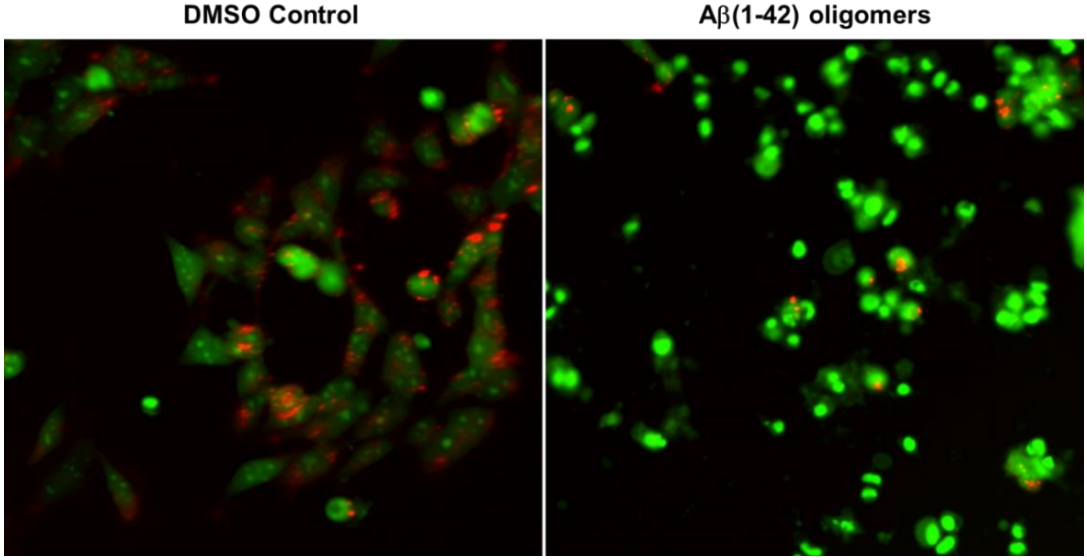
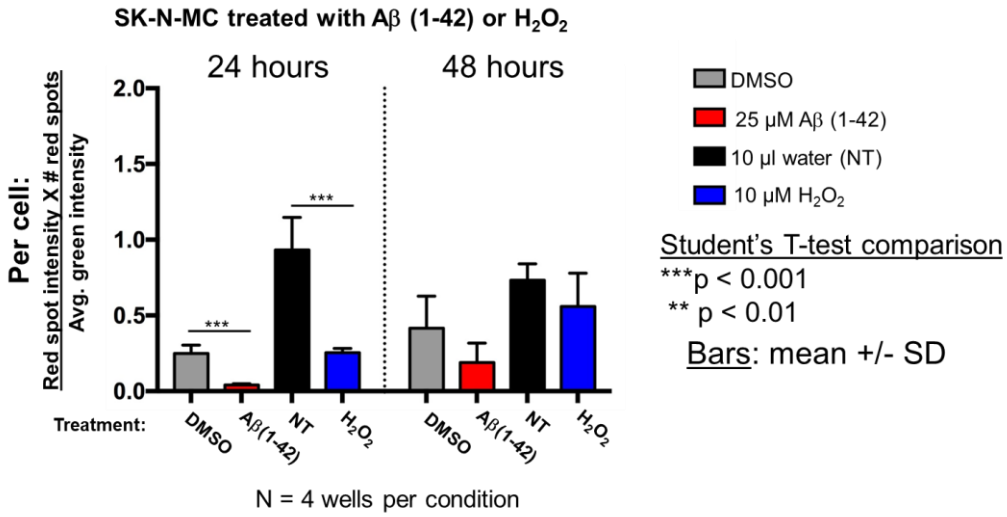
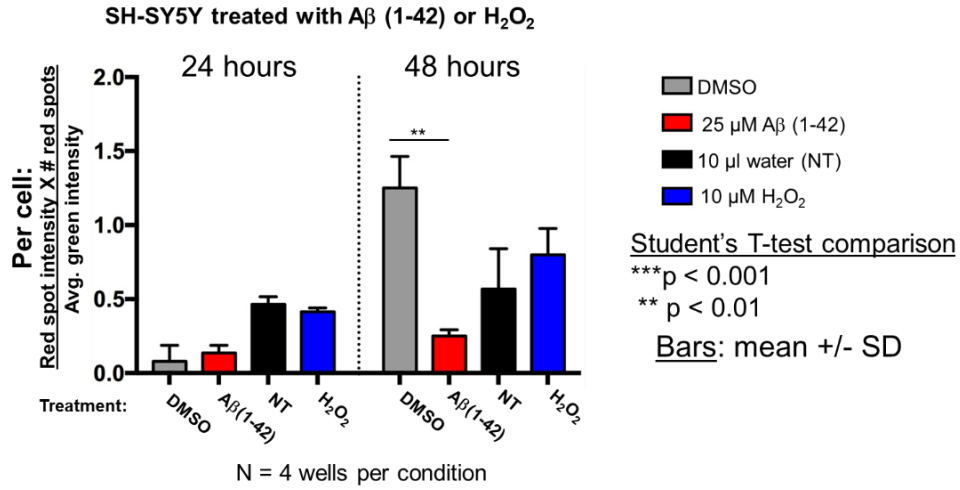


Figure 12. Example high-resolution image using acridine orange staining in SK-N-MC cells. The images are examples of representative high-resolution images obtained from acridine orange staining in SK-N-MC neuroblastoma cells. The left image is an example image obtained from the DMSO control and the right image is obtained after Aβ42 oligomer treatment. The green fluorescence indicates areas outside of the lysosome, and the red fluorescence indicates intact lysosomes. Thus, a decrease in red fluorescence indicates lysosomal leakage. Visually, it is observed that there is more red fluorescence in the DMSO control image compared to the Aβ42 oligomer treatment image.



Lower levels = lysosomal leakage

Figure 13. AO result of SK-N-MC cells treated with Aβ42 oligomers.
The bar graph shows the red fluorescence intensity per cell from the acridine orange (AO) staining fluorescence results for SK-N-MC neuroblastoma cells treated with Aβ42 oligomers or H_2O_2 compared to their respective controls of DMSO or water (NT), 24 and 48 hours after treatment. The red fluorescence intensity per cell is calculated by the equation: $\frac{(Red\ spot\ intensity) \times (\#\ of\ spots)}{(Average\ green\ intensity)}$. Lower levels indicate increased extent of lysosomal leakage. Treatment with Aβ42 oligomers showed about a significant five-fold decrease 24 hours after treatment compared to the DMSO control. Treatment with H_2O_2 showed about a three-fold decrease compared to the control, 24 hours after treatment.



Lower levels = lysosomal leakage

Figure 14. AO result of SH-SY5Y cells treated with Aβ42 oligomers.
The bar graph shows the red fluorescence intensity per cell from the acridine orange (AO) staining fluorescence results for SH-SY5Y neuroblastoma cells treated with Aβ42 oligomers or H_2O_2 compared to their respective controls of DMSO or water (NT), 24 and 48 hours after treatment. The red fluorescence intensity per cell is calculated by the equation: $\frac{(Red\ spot\ intensity) \times (\#\ of\ spots)}{(Average\ green\ intensity)}$. Lower levels indicate increased extent of lysosomal leakage. Treatment with Aβ42 oligomers showed about a significant five-fold decrease 48 hours after treatment compared to the DMSO control.

Aβ42 oligomers induce translocation of lysosomal cathepsin B to the cytosol

The result from the western blot analysis of BV2 microglial cells is shown in figure 15. By analyzing the cytosolic fraction samples obtained from sucrose gradient fractionation, it shows that when the BV2 microglial cells were treated with Aβ42, there are visible bands at around 43 and 31 kDa. In contrast, no bands are visible in the control with no Aβ42 treatment.

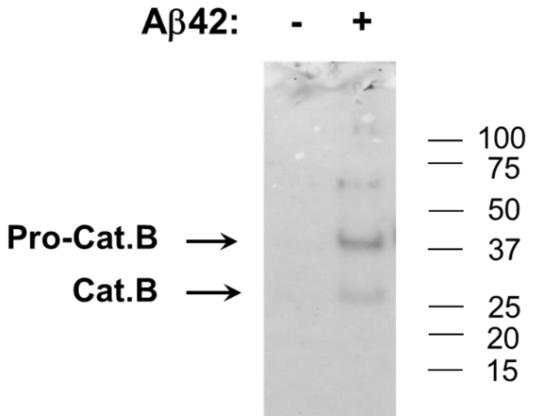


Figure 15. Aβ42 induces translocation of lysosomal CatB to the cytosol in BV2 cells.
The western blot above shows evidence of the translocation of lysosomal cathepsin B (CatB) into the cytosol of BV2 microglial cells as a result of Aβ42 oligomer treatment. The loaded samples are cytosolic fractions that were isolated and obtained via sucrose gradient fractionation. No bands were detected in the cytosolic fraction with no Aβ42 treatment. However, with Aβ42 treatment, bands at around 43 and 31 kilodaltons (kDa) are visible, indicating the presence of pro-CatB and mature CatB, respectively.

Aβ42 oligomers activate cysteine cathepsin activity at cytosolic neutral pH

An example fluorescent image obtained from SH-SY5Y neuroblastoma cells using the LES12 bifunctional probe is shown in figure 16. The results using the LES12 bifunctional probe in SH-SY5Y neuroblastoma cells is shown in figure 17. In SH-SY5Y cells, using the LES12 bifunctional probe showed that there was about a two-fold significant fold increase in cysteine cathepsin activity based on the average fluorescent intensity 24 hours after Aβ42 treatment. Moreover, there was a significant three-fold increase in neutral pH localization compared to the DMSO control 24 hours after treatment.

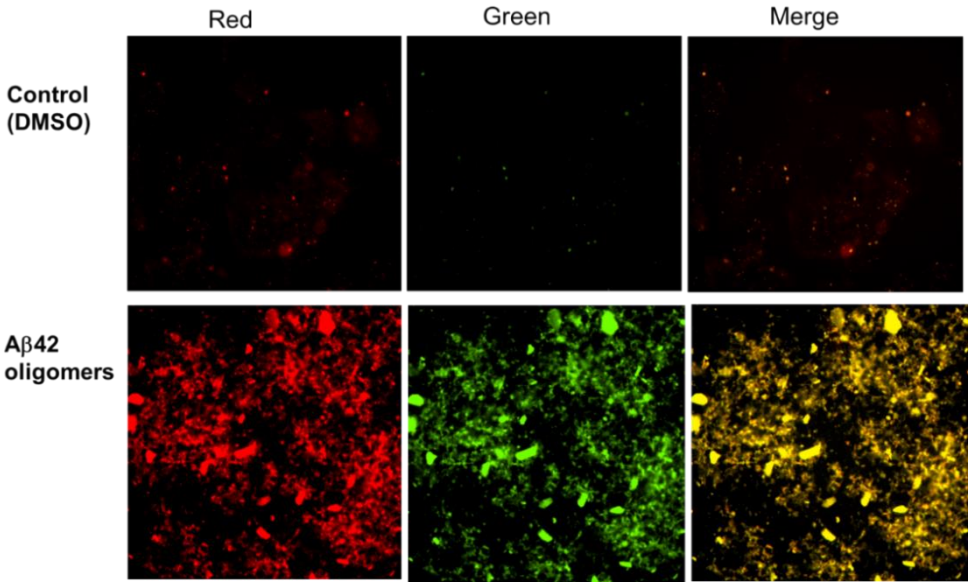


Figure 16. Example image using LES12 bifunctional probe in SH-SY5Y cells.
The images show an example image obtained using the LES12 bifunctional probe in SH-SY5Y neuroblastoma cells. The top set of images are obtained from the DMSO control, and the bottom set of images of are obtained 48 hours after Aβ42 oligomer treatment. The red fluorescence indicates the cysteine protease activity and the green fluorescence indicates the environment of the probe to be at around a neutral pH. When the red and green fluorescence images are merged, it results in a yellow fluorescence. This is indicative of protease activity at a neutral pH. Visually, it can be observed that compared to the DMSO control, Aβ42 oligomer treatment images show a greater fluorescence intensity overall, indicating a greater cysteine protease activity at neutral pH.

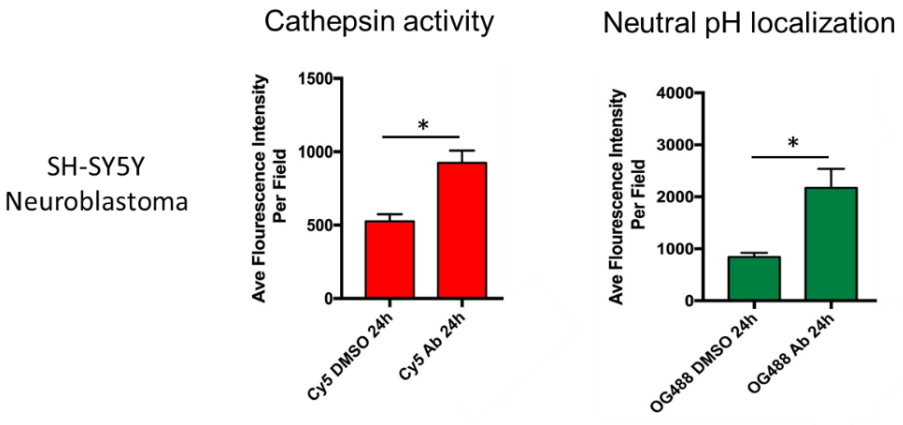


Figure 17. LES12 bifunctional probe result of SH-SY5Y cells treated with Aβ42 oligomers.
The bar graph shows the results of the average fluorescence intensity per field from the LES12 bifunctional probe used in SH-SY5Y neuroblastoma cells treated with Aβ42 oligomers compared to the DMSO control. The left bar graph shows the cysteine cathepsin activity and the right bar graph shows the neutral pH localization. Treatment with Aβ42 oligomers showed about a significant two-fold increase in cysteine cathepsin activity 24 hours after treatment compared to the DMSO control. Furthermore, there was a significant three-fold increase in neutral pH localization 24 hours after treatment compared to the DMSO control.

The results section is coauthored with Podvin, Sonia and Ito, Gen. The thesis author was the co-author of this section.

4. Discussion

By performing LDH assays, SK-N-MC and SH-SY5Y neuroblastoma cells both showed significant increase in NADH levels after treatment with Aβ42 oligomers. Moreover, fluorescence imaging from acridine orange staining revealed decreased red fluorescence intensities for both neuroblastoma cells. Lastly, LES bifunctional probe showed increased cysteine cathepsin activity and neutral pH localization in SH-SY5Y cells after Aβ42 oligomer treatment.

The LDH assay results show that for both SK-N-MC and SH-SY5Y neuroblastoma cells, treatment with Aβ42 oligomers results in cell cytotoxicity as indicated by the increased NADH levels compared to the DMSO control. This is as expected as a study by Wang et al. showed that Aβ42 induces cell death in SH-SY5Y neuroblastoma cells (2010). Moreover, another study showed cell apoptosis induced by Aβ42 in SH-SY5Y neuroblastoma cells as well (Huang and Liu, 2015).

With the confirmation of Aβ42 oligomers inducing cell cytotoxicity in the neuroblastoma cells, acridine orange staining was used to observe whether Aβ42 oligomer treatment would stimulate lysosomal leakage. The decrease in the red fluorescence intensity compared to the DMSO control indicate that there is lysosomal leakage. This result is consistent with a previous study that showed a loss in lysosomal membrane impermeability, in other words lysosomal leakage, in SH-SY5Y neuroblastoma cells after incubation with Aβ42 observed via Lucifer Yellow fluorescence monitoring (Yang et al., 1998).

The western blot analysis showed visible bands at around 43 and 31 kDa when the BV2 microglial cells were treated with Aβ42. According to Cavallo-Medved et al., these bands would represent pro-CatB and mature CatB, respectively, as the molecular weight for pro-CatB is said to be 43 kDa, and 31 kDa for mature CatB. These results shows that there is Aβ42-induced translocation of lysosomal CatB to the cytosol. This is evident due to the fact that the analysis was done on cytosolic fraction samples, meaning that the observed CatB is present in the

cytosol. Moreover, this translocation of CatB was induced by Aβ42 oligomers since no CatB was present in the control cytosolic fraction samples.

The findings of cysteine cathepsin activity and neutral pH localization using the LES 12 bifunctional probe is important as it shows a relatively new method used in the field to look at the activity of cysteine cathepsins in a neutral pH, which is representative of the cytosol. Though the results do not show specificity in the activity of what cysteine cathepsins are involved, it is a valid first step in verifying general cysteine cathepsin activity in the cytosol, induced by Aβ42 oligomers. This will justify further experiments to look into specific cysteine cathepsin activity, such as CatB, as it has been thought to cause cellular damage when translocated into the cytosol from the lysosome due to the preservation of its enzymatic activity in neutral pH (Hook et al., 2015).

5. Conclusion

The findings from the experiments from the LDH assays and AO staining show and confirm the induction of lysosomal leakage and cell death as a result of Aβ42 oligomer treatment in SK-N-MC and SH-SY5Y human neuroblastoma cells. Moreover, the western blot analysis showed that Aβ42 induces the translocation of lysosomal CatB into the cytosol in BV2 microglial cells. Furthermore, the activity of cysteine cathepsins in cytosolic neutral pH is observed in SH-SY5Y human neuroblastoma cells, as evident with the LES12 bifunctional probe experiment.

It can be concluded that Aβ42 oligomers induce lysosomal leakage, as well as cell death. In addition, the Aβ42-induction of CatB translocation from the lysosomes to the cytosol is observed. Lastly, cysteine cathepsin activity in a cytosolic neutral pH environment is demonstrated. The next steps in this project can be to look at the activity of CatB in cell homogenates, as well as in the cytosolic fractions obtained through sucrose gradient fractionation. Moreover, the CatB inhibitor, CA-074 can be utilized to look at its effects on CatB

activity in lysosomes as well as in the cytosol. Finally, the amount of cysteine cathepsins in cells with and without Aβ42 treatment can be looked into through proteomics. The findings from this study, along with future experiments will further the mechanistic understanding of Aβ42-induced lysosomal leakage of CatB in the neuropathology of AD and other related neurodegenerative diseases, ultimately allowing for the discovery of potential therapeutic targets.

F. Significance of new results

The new results obtained from the experiments advances the understanding of lysosomal leakage and CatB in AD and other related neurodegenerative diseases. The data from the LDH assay and the AO staining showed that Aβ42 oligomers induce cell cytotoxicity and stimulates lysosomal leakage. Moreover, the western blot analysis showed that Aβ42 induces the translocation of CatB into the cytosol. Lastly, the LES12 bifunctional probe showed that cysteine cathepsins are active in the cytosolic neutral pH environment. Piecing these results together, it shows that Aβ42 stimulates lysosomal leakage, resulting in the translocation of CatB into the cytosol. This ultimately results in cell death, which can be attributed to the activity of cysteine cathepsin, such as CatB in the cytosol. This indication that CatB plays a role in cell death validates CatB as a potential therapeutic target for AD and other related neurodegenerative diseases.

G. References

2020 Alzheimer's disease facts and figures. Alzheimer’s Dement. 2020 Mar 10. doi: 10.1002/alz.12068. Epub ahead of print. PMID: 32157811.

Cavallo-Medved D, Moin K, Sloane B. Cathepsin B: Basis Sequence: Mouse. AFCS Nat Mol Pages. 2011;2011:A000508. Epub 2011 Apr 10. PMID: 28781583; PMCID: PMC5541861.

Chen S, Zhou C, Yu H, Tao L, An Y, Zhang X, Wang Y, Wang Y, Xiao R. 27-Hydroxycholesterol Contributes to Lysosomal Membrane Permeabilization-Mediated Pyroptosis in Co-cultured SH-SY5Y Cells and C6 Cells. Front Mol Neurosci. 2019 Mar 1;12:14. doi: 10.3389/fnmol.2019.00014. PMID: 30881285; PMCID: PMC6405519.

De Kimpe L, van Haastert ES, Kaminari A, Zwart R, Rutjes H, Hoozemans JJ, Scheper W. Intracellular accumulation of aggregated pyroglutamate amyloid beta: convergence of aging and Aβ pathology at the lysosome. Age (Dordr). 2013 Jun;35(3):673-87. doi: 10.1007/s11357-012-9403-0. Epub 2012 Apr 4. PMID: 22477259; PMCID: PMC3636379.

Ditaranto K, Tekirian TL, Yang AJ. Lysosomal membrane damage in soluble Abeta-mediated cell death in Alzheimer's disease. Neurobiol Dis. 2001 Feb;8(1):19-31. doi: 10.1006/nbdi.2000.0364. PMID: 11162237.

Fassio A, Falace A, Esposito A, Aprile D, Guerrini R, Benfenati F. Emerging Role of the Autophagy/Lysosomal Degradative Pathway in Neurodevelopmental Disorders with Epilepsy. Front Cell Neurosci. 2020 Mar 13;14:39. doi: 10.3389/fncel.2020.00039. PMID: 32231521; PMCID: PMC7082311.

Felbor U, Kessler B, Mothes W, Goebel HH, Ploegh HL, Bronson RT, Olsen BR. Neuronal loss and brain atrophy in mice lacking cathepsins B and L. Proc Natl Acad Sci U S A. 2002 Jun 11;99(12):7883-8. doi: 10.1073/pnas.112632299. Epub 2002 Jun 4. PMID: 12048238; PMCID: PMC122989.

Gabandé-Rodríguez E, Pérez-Cañamás A, Soto-Huelin B, Mitroi DN, Sánchez-Redondo S, Martínez-Sáez E, Venero C, Peinado H, Ledesma MD. Lipid-induced lysosomal damage after demyelination corrupts microglia protective function in lysosomal storage disorders. EMBO J. 2019 Jan 15;38(2):e99553. doi: 10.15252/embj.201899553. Epub 2018 Dec 7. PMID: 30530526; PMCID: PMC6331723.

Gan L, Ye S, Chu A, Anton K, Yi S, Vincent VA, von Schack D, Chin D, Murray J, Lohr S, Patthy L, Gonzalez-Zulueta M, Nikolich K, Urfer R. Identification of cathepsin B as a mediator of neuronal death induced by Abeta-activated microglial cells using a functional genomics approach. J Biol Chem. 2004 Feb 13;279(7):5565-72. doi: 10.1074/jbc.M306183200. Epub 2003 Nov 10. Erratum in: J Biol Chem. 2004 May 28;279(22):23845. PMID: 14612454.

GBD 2016 Neurology Collaborators. Global, regional, and national burden of neurological disorders, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2019 May;18(5):459-480. doi: 10.1016/S1474-4422(18)30499-X. Epub 2019 Mar 14. PMID: 30879893; PMCID: PMC6459001.

Grunwald D. Flow cytometry and RNA studies. Biol Cell. 1993;78:27-30. doi: 10.1016/0248-4900(93)90111-Q.

Hickman S, Izzy S, Sen P, Morsett L, El Khoury J. Microglia in neurodegeneration. Nat Neurosci. 2018 Oct;21(10):1359-1369. doi: 10.1038/s41593-018-0242-x. Epub 2018 Sep 26. PMID: 30258234; PMCID: PMC6817969.

Hook G, Jacobsen JS, Grabstein K, Kindy M, Hook V. Cathepsin B is a New Drug Target for Traumatic Brain Injury Therapeutics: Evidence for E64d as a Promising Lead Drug Candidate. Front Neurol. 2015 Sep 2;6:178. doi: 10.3389/fneur.2015.00178. PMID: 26388830; PMCID: PMC4557097.

Hook V, Yoon M, Mosier C, Ito G, Podvin S, Head BP, Rissman R, O'Donoghue AJ, Hook G. Cathepsin B in neurodegeneration of Alzheimer's disease, traumatic brain injury, and related brain disorders. Biochim Biophys Acta Proteins Proteom. 2020 Aug;1868(8):140428. doi: 10.1016/j.bbapap.2020.140428. Epub 2020 Apr 17. PMID: 32305689; PMCID: PMC7261628.

Hsu A, Podvin S, Hook V. Lysosomal Cathepsin Protease Gene Expression Profiles in the Human Brain During Normal Development. J Mol Neurosci. 2018 Aug;65(4):420-431. doi: 10.1007/s12031-018-1110-6. Epub 2018 Jul 14. PMID: 30008074; PMCID: PMC7185200.

Huang Y, Liu T. Amyloid Beta Peptide 1-42 Induces SH-SY5Y Cell Apoptosis via the Promotion of Meg3 Long Noncoding RNA Expression. Integr Med Int. 2015;2:73-79. doi: 10.1159/000438702

Ji ZS, Miranda RD, Newhouse YM, Weisgraber KH, Huang Y, Mahley RW. Apolipoprotein E4 potentiates amyloid beta peptide-induced lysosomal leakage and apoptosis in neuronal cells. J Biol Chem. 2002 Jun 14;277(24):21821-8. doi: 10.1074/jbc.M112109200. Epub 2002 Mar 23. PMID: 11912196.

Kindy MS, Yu J, Zhu H, El-Amouri SS, Hook V, Hook GR. Deletion of the cathepsin B gene improves memory deficits in a transgenic ALZHeimer's disease mouse model expressing AβPP containing the wild-type β-secretase site sequence. J Alzheimers Dis. 2012;29(4):827-40. doi: 10.3233/JAD-2012-111604. PMID: 22337825; PMCID: PMC4309289.

Kingham PJ, Pocock JM. Microglial secreted cathepsin B induces neuronal apoptosis. J Neurochem. 2001 Mar;76(5):1475-84. doi: 10.1046/j.1471-4159.2001.00146.x. PMID: 11238732.

Lopez-Castejon G, Brough D. Understanding the mechanism of IL-1β secretion. Cytokine Growth Factor Rev. 2011 Aug;22(4):189-95. doi: 10.1016/j.cytogfr.2011.10.001. Epub 2011 Oct 22. PMID: 22019906; PMCID: PMC3714593.

Lowry JR, Klegeris A. Emerging roles of microglial cathepsins in neurodegenerative disease. Brain Res Bull. 2018 May;139:144-156. doi: 10.1016/j.brainresbull.2018.02.014. Epub 2018 Feb 15. PMID: 29454581.

Man SM, Kanneganti TD. Regulation of lysosomal dynamics and autophagy by CTSB/cathepsin B. Autophagy. 2016 Dec;12(12):2504-2505. doi: 10.1080/15548627.2016.1239679. Epub 2016 Oct 27. PMID: 27786577; PMCID: PMC5173259.

Mort JS, Buttle DJ. Cathepsin B. Int J Biochem Cell Biol. 1997 May;29(5):715-720. doi: 10.1016/S1357-2725(96)00152-5.

Ni J, Wu Z, Stoka V, Meng J, Hayashi Y, Peters C, Qing H, Turk V, Nakanishi H. Increased expression and altered subcellular distribution of cathepsin B in microglia induce cognitive impairment through oxidative stress and inflammatory response in mice. Aging Cell. 2019 Feb;18(1):e12856. doi: 10.1111/acel.12856. Epub 2018 Dec 21. PMID: 30575263; PMCID: PMC6351837.

Nixon RA. Amyloid precursor protein and endosomal-lysosomal dysfunction in Alzheimer's disease: inseparable partners in a multifactorial disease. FASEB J. 2017 Jul;31(7):2729-2743. doi: 10.1096/fj.201700359. PMID: 28663518; PMCID: PMC6137496.

Racoma IO, Meisen WH, Wang QE, Kaur B, Wani AA. Thymoquinone inhibits autophagy and induces cathepsin-mediated, caspase-independent cell death in glioblastoma cells. PLoS One. 2013 Sep 9;8(9):e72882. doi: 10.1371/journal.pone.0072882. PMID: 24039814; PMCID: PMC3767730.

Sambri I, D'Alessio R, Ezhova Y, Giuliano T, Sorrentino NC, Cacace V, De Risi M, Cataldi M, Annunziato L, De Leonibus E, Fraldi A. Lysosomal dysfunction disrupts presynaptic maintenance and restoration of presynaptic function prevents neurodegeneration in lysosomal storage diseases. EMBO Mol Med. 2017 Jan;9(1):112-132. doi: 10.15252/emmm.201606965. PMID: 27881461; PMCID: PMC5210158.

Sanman LE, van der Linden WA, Verdoes M, Bogyo M. Bifunctional Probes of Cathepsin Protease Activity and pH Reveal Alterations in Endolysosomal pH during Bacterial Infection. Cell Chem Biol. 2016 Jul 21;23(7):793-804. doi: 10.1016/j.chembiol.2016.05.019. Epub 2016 Jul 14. PMID: 27427229; PMCID: PMC4982764.

Sarkar C, Jones JW, Hegdekar N, Thayer JA, Kumar A, Faden AI, Kane MA, Lipinski MM. PLA2G4A/cPLA2-mediated lysosomal membrane damage leads to inhibition of autophagy and neurodegeneration after brain trauma. Autophagy. 2020 Mar;16(3):466-485. doi: 10.1080/15548627.2019.1628538. Epub 2019 Jun 25. PMID: 31238788; PMCID: PMC6999646.

Taneo J, Adachi T, Yoshida A, Takayasu K, Takahara K, Inaba K. Amyloid β oligomers induce interleukin-1β production in primary microglia in a cathepsin B- and reactive oxygen species-dependent manner. Biochem Biophys Res Commun. 2015 Mar 13;458(3):561-567. doi: 10.1016/j.bbrc.2015.02.006. Epub 2015 Feb 11. PMID: 25680460.

Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. Int J Nanomedicine. 2019 Jul 19;14:5541-5554. doi: 10.2147/IJN.S200490. PMID: 31410002; PMCID: PMC6650620.

Vermes I, Haanen C. Apoptosis and programmed cell death in health and disease. Adv Clin Chem. 1994;31:177-246. doi: 10.1016/s0065-2423(08)60336-4. PMID: 7879672.

Wang F, Gómez-Sintes R, Boya P. Lysosomal membrane permeabilization and cell death. Traffic. 2018 Dec;19(12):918-931. doi: 10.1111/tra.12613. Epub 2018 Sep 12. PMID: 30125440.

Wang H, Ma J, Tan Y, Wang Z, Sheng C, Chen S, Ding J. Amyloid-beta1-42 induces reactive oxygen species-mediated autophagic cell death in U87 and SH-SY5Y cells. J Alzheimers Dis. 2010;21(2):597-610. doi: 10.3233/JAD-2010-091207. PMID: 20571221.

Wu Z, Ni J, Liu Y, Teeling JL, Takayama F, Collcutt A, Ibbett P, Nakanishi H. Cathepsin B plays a critical role in inducing Alzheimer's disease-like phenotypes following chronic systemic exposure to lipopolysaccharide from Porphyromonas gingivalis in mice. Brain Behav Immun. 2017 Oct;65:350-361. doi: 10.1016/j.bbi.2017.06.002. Epub 2017 Jun 10. PMID: 28610747.

Xu M, Yang L, Rong JG, Ni Y, Gu WW, Luo Y, Ishidoh K, Katunuma N, Li ZS, Zhang HL. Inhibition of cysteine cathepsin B and L activation in astrocytes contributes to neuroprotection against cerebral ischemia via blocking the tBid-mitochondrial apoptotic signaling pathway. Glia. 2014 Jun;62(6):855-80. doi: 10.1002/glia.22645. Epub 2014 Feb 24. PMID: 24616078.

Yadati T, Houben T, Bitorina A, Shiri-Sverdlov R. The Ins and Outs of Cathepsins: Physiological Function and Role in Disease Management. Cells. 2020 Jul 13;9(7):1679. doi: 10.3390/cells9071679. PMID: 32668602; PMCID: PMC7407943.

Yan M, Zhu W, Zheng X, Li Y, Tang L, Lu B, Chen W, Qiu P, Leng T, Lin S, Yan G, Yin W. Effect of glutamate on lysosomal membrane permeabilization in primary cultured cortical neurons. Mol Med Rep. 2016 Mar;13(3):2499-505. doi: 10.3892/mmr.2016.4819. Epub 2016 Jan 28. PMID: 26821268; PMCID: PMC4768955.

Yang AJ, Chandswangbhuvana D, Margol L, Glabe CG. Loss of endosomal/lysosomal membrane impermeability is an early event in amyloid Abeta1-42 pathogenesis. J Neurosci Res. 1998 Jun 15;52(6):691-8. doi: 10.1002/(SICI)1097-4547(19980615)52:6<691::AID-JNR8>3.0.CO;2-3. PMID: 9669318.