**The Effect of β-amyloid on Thioredoxin Antioxidant System**

By

**Yiran Wang**

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

**MASTER OF SCIENCE**

Department of Pharmacology and Therapeutics

University of Manitoba

Winnipeg, Canada

## ABSTRACT

Beta-amyloid peptide (Aβ) contributes significantly to neurodegeneration during the development of Alzheimer's disease (AD). Many studies showed that Aβ can cause oxidative damage. Thioredoxin (Trx) is a redox protein that can reverse cysteine oxidative modifications such as sulfenylation and nitrosylation. Thioredoxin-interacting protein (Txnip) is an endogenous Trx inhibitor. In the present study, using immunoblotting analysis, I found that Txnip protein levels and total sulfenylated protein levels were significantly increased in the hippocampus and frontal cortex of 9 and 12-month old *App/Ps1* mice compared with wild-type mice. Aβ also directly increased Txnip protein levels, and cysteine sulfenylation and nitrosylation. Using CRISPR/Cas9 technology, we found that knocking out Txnip in HT22 cells blocked Aβ-induced protein sulfenylation and nitrosylation. Our findings suggest that Aβ may upregulate Txnip, subsequently inhibiting Trx activity and enhancing cysteine sulfenylation and nitrosylation. These results also indicate that Txnip may have potential for AD treatment.

## ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my advisor Dr. Jun-Feng Wang for the continuous support of my graduate study and research in neuroscience, for his patience, motivation, and immense knowledge. His guidance helped me through three important years of my life.

I would also like to appreciate Dr. Tiina Kauppinen and Dr. Tabrez Siddiqui to be on my thesis committee despite their extremely busy schedule. I am tremendously grateful for their guidance and support on my project, and suggestions on my thesis writing. Without their efforts and kindness, this work would not have been done.

And I would like to thank all my colleagues who helped me get through three years of graduate school. Our research associate Dr. Hua Tan, thank you for every convenience you gave me and sharing your valuable technical experiences. Also, I would like to thank the graduate student Ying Wang for helping me arrange everything when I first came to Winnipeg. I would like to thank the graduate students Ying Wang, Veni Bharti, Hong Zhou and our summer student Vanessa Hoi for helping me with experiments. It is a great experience to work with all of you!

I would like to thank our collaborator Dr. Eftekhar Eftekharpour for providing us the transfected cells by CRISPR/Ca9. I also would like to thank Dr. Michael Jackson for providing us the mouse cerebral cortex as a free gift.

At the very end, I would like to appreciate my parents for their love and unconditional support during my study in Canada. Many thanks go out to my friends in the University – Yujiao Xing, Xuan Chen, Reiko Roppongi, Xinwen Dong, Selina Chi, Jiaying You, Jiaqi Yang, Xiaodan Jiao. I would also like to appreciate my best friend Yuhua Fang for his endless courage and patience during my stay in Canada. These individuals always helped me to keep my life in context.

This project is supported by research funding from the National Sciences and Engineering Research Council Discovery Grant.

## TABLES OF CONTENTS

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

| | |
|---|---|
| 4-HNE | 4-hydroxyhexenal |
| 8-OH-dG | 8-hydroxy-2'-deoxyguanosine |
| AD | Alzheimer's disease |
| AICD | Amyloid Precursor Protein Intracellular Domain |
| AP-1 | activator protein 1 |
| ApoE | apolipoprotein E |
| APP | Amyloid Precursor Protein |
| APPsα | Soluble Amyloid Precursor Protein α |
| APPsβ | Soluble Amyloid Precursor Protein β |
| ARE | antioxidant response element |
| ASK1 | apoptosis signal-regulating kinase 1 |
| Aβ | β-amyloid |
| BACE1 | β-site APP-Cleaving Enzyme |
| bZip | basic region leucine zipper |
| Cdk5 | cyclin-dependent kinase 5 |
| CSF | cerebrospinal fluid |
| Cys | Cysteine |
| DJ-1 | deglycase |
| GSK-3β | glycogen synthase kinase 3β |
| GSNO | S-nitrosoglutathione |
| IDE | insulin-degrading enzyme |
| IL-18 | interleukin-18 |
| IL-1$\beta$ | interleukin-1β |
| JNK | c-Jun N-terminal kinase |
| MAPK | mitogen-activated protein kinase |
| MAPKKK | mitogen-activated protein kinase kinase kinase |
| MCI | mild cognitive impairment |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NLRP3 | nod-like receptor protein 3 |
| NMDA | N-methyl-D-aspartate |
| NO | nitric oxide |
| NOS | nitric oxide synthase |
| Nrf2 | nuclear factor erythroid 2 (NFE2)-related factor 2 |
| NSCs | neural stem cells |
| P38 | P38 mitogen-activated kinase |
| PHF | paired helical filaments |

| | |
|---|---|
| Prx | peroxiredoxin |
| PS | presenilin |
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| Sec | selenocysteine |
| SF | straight filaments |
| SOD | superoxide dismutase |
| Trx | thioredoxin |
| TrxR | thioredoxin reductase |
| Txnip | Thioredoxin-interacting protein |
| α-CTF | α-carboxyl-terminal fragment |
| β-CTF | β-secretase-cleaved C terminal fragment |

# CHAPTER 1: INTRODUCTION

## 1.1 Beta-amyloids and Alzheimer's Disease

### 1.1.1 Neurobiology of Alzheimer's disease

Alzheimer's disease (AD) was first identified in 1906 by a German psychiatrist Alois Alzheimer and used to describe the case of his patient Auguste D who showed profound memory loss, unfounded suspicions about her family, and other worsening psychological changes. Over the last century, AD has become known as the most common cause of dementia. An estimated 5.5 million Americans are living with AD in 2017, a number projected to grow to 13.8 million by mid-21$^{st}$ century [1]. AD is recognized as a neurodegenerative brain disease with symptoms of memory loss, declined cognitive function and behavior changes such as apathy, agitation and aggression. The highest risk factors for AD include aging, family history with AD and carrying the apolipoprotein E (ApoE) ε4 gene. Although clear mechanisms have not yet been explained, amyloid plaques, neurofibrillary tangles and deficency of cholinergic and glutamergic neurotransmission have been considered as main pathological factors in AD.

The amyloid hypothesis postulates that neurodegeneration in AD is caused by abnormal accumulation of β-amyloid plaques in various areas of the brain. β-amyloid peptide (Aβ), the main component of amyloid plaques, plays a critical role in the origin and progression of the nervous tissue damage in AD patients. Aβ was first sequenced

from the meningeal blood vessels of patients suffering from AD in 1984 [2]. Subsequently, Aβ was confirmed in the amyloid plaques of AD patient brain tissue [3][4]. Hardy and Allsop formally proposed the amyloid hypothesis for the first time in 1991 [5]. Shortly after studies found that amyloid precursor protein (APP), presenilin (PS1) and PS2 gene mutations were associated with AD development [6][7][8]. Nowadays it is widely believed that mutations in these genes can induce increased production of Aβ peptides. Further studies demonstrated that the aggregation of Aβ could be considered as the primary pathological trigger contributing to neuritic injury, neuronal dysfunction, and cell death in AD [9][10].

Neurofibrillary tangles are caused by hyperphosphorylation of Tau proteins. Belonging to the family of microtubule-associated proteins, Tau proteins are mainly located in neurons [11] and play a central role in assembling and stabilizing neuronal microtubule network under physiological conditions [12]. The binding activity between tau protein and microtubule can be regulated post-translationally by different kinases such as glycogen synthase kinase 3β (GSK-3β), cyclin-dependent kinase (Cdk5) or mitogen-activated protein kinase (MAPK). The hyperphosphorylation of tau reduces its affinity towards microtubules. Under pathological conditions, increased levels of hyperphosphorylated tau proteins detach from microtubules and accumulate in the cytosol [13]. Further, detached hyperphosphorylated tau protein polymerizes into paired

helical filaments (PHF) and straight filaments (SF) referred to as neurofibrillary tangles [14]. It was found that increased levels of hyperphosphorylated tau protein accumulated in the neuronal cytosol of AD brain [15]. The loss of normal tau protein function will affect cellular functions of neurons, such as maintenance of appropriate morphology, axonal transport, synaptic dysfunction and neurodegeneration [16].

Cholinergic dysfunction is the oldest hypothesis in AD. The degeneration of cholinergic neurons in the basal forebrain is thought to be one of the earliest pathological events in AD patients [1][16][18]. Cholinergic deficits lead to the decreased synthesis of acetylcholine. In the brain, acetylcholine functions as an essential neurotransmitter in central nervous system and peripheral nervous system, modulating cognitive processes such as arousal, attention, and memory. Choline acetyltransferase is responsible for the synthesis of the neurotransmitter acetylcholine. Studies reported the reduction of choline acetyltransferase activity in the cerebral cortex of brain tissues from AD patients [17][18]. *In vitro* studies have shown that Aβ inhibits cholinergic neurotransmission [19][20]. It has been found that cholinergic dysfunction may also contribute to the formation of Aβ plaques [21].

Glutamate is the major excitatory neurotransmitter, accounting for over 90% of the synaptic connections in the human brain [131]. The two main categories of glutamate receptors are AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and

NMDA (N-methyl-D-aspartate) receptors. AMPA receptors are ionotropic receptors which are permeable to $Na^+$, $K^+$ and to a small extent $Ca^{2+}$, and specialized for fast excitation. NMDA receptors are also ionotropic but mainly permeable to $Ca^{2+}$, and are particularly important for learning and memory when activated [131][132]. Constant glutamate signaling is required to activate long-term potentiation, which is a crucial hypothesis of long-term memory [133]. In AD, chronic mild activation of NMDA receptors ultimately leading to neurodegeneration is one of the leading symptoms of neurotransmission imbalance termed excitotoxicity [133]. Hyperactivation of NMDA receptors allows the influx of Ca2+ ions into postsynaptic neurons, leading to increased nitric oxide (NO) production, increased levels of free radicals, dysfunction of calcium homeostasis, activation of proteases, increased cytotoxic transcription factors and neuronal cell death [134].

### 1.1.2 Formation of Aβ

Aβ is composed of 39-43 amino acids that are proteolytically derived from the sequential enzymatic cleavage of the widely distributed transmembrane APP by β-secretase and γ-secretase [6][24][151]. The APP gene is located on chromosome 21. Alternative splicing of APP transcript generates eight isoforms with length ranging from 639 to 770 amino acids [152][153]. Among these isoforms, the 695, 751 and 770 amino

acid forms are the most common [161]. The 695 amino acid form is predominantly expressed in the central nervous system, while 751 and 770 amino acid forms are ubiquitously expressed in the skin [153][154]. In neurons, APP is produced in a large quantity and metabolized very rapidly [154]. After sorting through the endoplasmic reticulum and Golgi, APP is delivered by fast axonal transport to synaptic terminals [153][155]. Critical steps of APP processing occur in the trans-Golgi network and cell surface, generating various products with different functions [22].

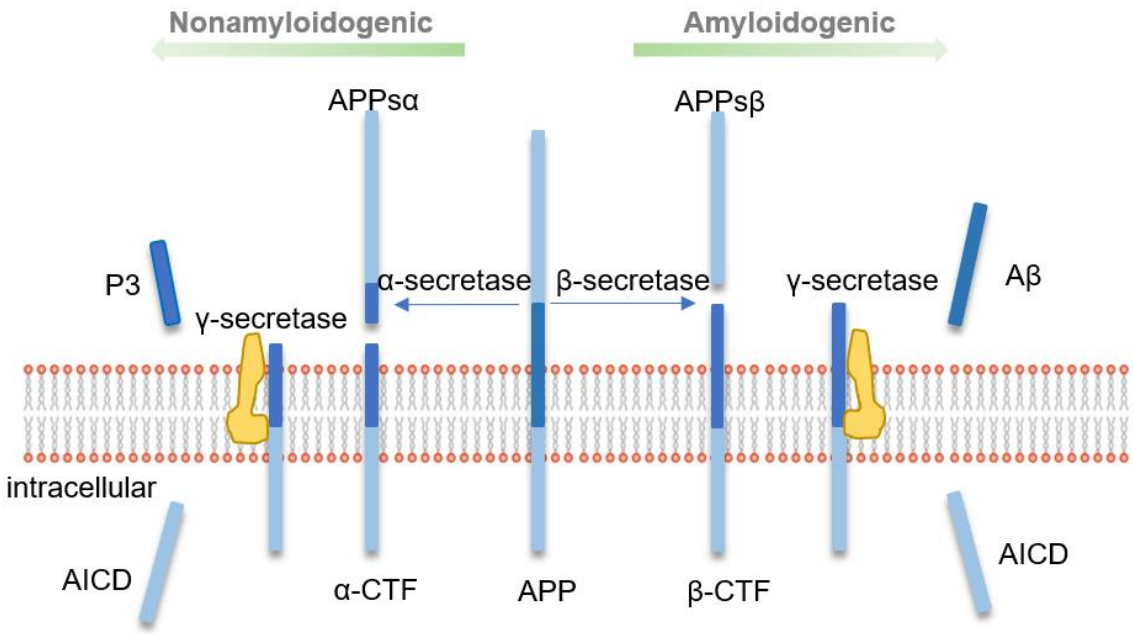


**Figure 1. The structure and metabolism of amyloid precursor protein (APP) by two processing pathways.** The nonamyloidogenic processing of APP involving α-secretase followed by γ-secretase (left); the amyloidogenic processing of APP involving β-secretase followed by γ-secretase (right). Abbreviations: APP, Amyloid Precursor Protein; APPsα, Soluble Amyloid Precursor Protein α; APPsβ, Soluble Amyloid Precursor Protein β; αCTF, α-carboxyl-terminal fragment; βCTF, β-secretase-cleaved C terminal fragment; AICD, Amyloid Precursor Protein Intracellular Domain.

Sequential cleavage of APP occurs by two pathways (Figure 1) . The first pathway is nonamyloidogenic processing of APP proteolyzed by α-secretase followed by γ-secretase [151][153]. The cleavage of APP by α-secretase results in the production of a soluble form of APP (APPsα) and α-carboxyl-terminal fragment (α-CTF) [154]. α-CTF is further proteolyzed by γ-secretase to produce small peptide P3 and amyloid precursor protein intracellular domain (AICD) [154][157]. The APPsα shows functions similar to growth factors and increases proliferation of embryonic neural stem cells (NSCs) *in vitro* [161]. In contrast to the role of APPsα in proliferation, AICD has been suggested to be a negative regulator of neural progenitor cells proliferation [157]. P3 fragment (Aβ 17-40/42) has less toxic effect than Aβ1-42. The second pathway is an amyloidogenic pathway. In this pathway, APP is initially cleaved by β-secretase (also known as aspartyl protease BACE1) to produce a soluble secreted form of APP (APPsβ) and a C-terminal fragment β-CTF [25][158]. The subsequent cleavage of β-CTF by γ-secretase yields Aβ peptide and AICD [155][156]. APPsβ is reported to be a death receptor ligand, mediating axonal pruning and neuronal cell death [154][160]. The length of Aβ peptide varies at the C-terminal according to the cleavage pattern by γ-secretase [156]. Major Aβ peptide products are Aβ1–40 and Aβ1–42. Aβ1–40 is the most prevalent [162]. However, Aβ1–42 monomers are more prone to being aggregated and assembled into potential toxic oligomers under pathological conditions [154][162]. It has been reported that Aβ

oligomers can cause synapse loss and neuronal death by disrupting various signaling pathways like fyn kinase, GSK3β, and Cdk5 pathways [159][163]. It was also reported that Aβ can increase oxidative stress, hyperactivate N-methyl-D-aspartate (NMDA) receptors and impair mitochondrial function [164].

### 1.1.3 Aβ in Alzheimer's disease

Many studies have supported the pathological role of Aβ in AD. In AD patients, studies found that Aβ peptide is the principal component of Aβ plaques [23][24][136]. Studies have shown that mutations in APP and presenilins lead to higher amounts of the disease-linked Aβ aggregation [25]. Studies have also shown that the disease-associated Aβ42 peptides decline in cerebrospinal fluid (CSF) of AD patients approximately 1–2 decades before the onset of symptoms [26][27]. Recently, the toxic Aβ oligomer enriched with β-sheet has been primarily found to cause cytotoxicity, synaptic impairment and memory deficiency in rodents [28]. For these reasons, Aβ has been considered as a pathological trigger of neuronal dysfunction and cell death in AD.

### 1.1.4 *App/Ps1* Animal Model in AD Research

Currently there is no effective treatment for AD. Many studies on AD depend on appropriate animal models. Most animal models are established based on genetics,

mainly involving APP gene on chromosome 21, PS1 gene on chromosome 14, PS2 gene on chromosome 1, Tau protein gene on chromosome 17 and ApoE gene on chromosome 19 [29]. The *App/Ps1* double transgenic mouse model is one of the most commonly and extensively used in AD studies, due to the similar pathological changes to AD.

The first gene mutation identified as a cause of autosomal dominant AD was the APP gene [30]. APP mutations are named according to the geographic location from which the affected family originated. The K670N/M671L double mutation at the β-secretase cleavage site, initially found in a Swedish family, results in increased β-secretase cleavage and thus increased Aβ production of both Aβ40 and Aβ42 [6][31]. Mutations in the presenilin (PS) genes are another cause of autosomal dominant AD [32][8]. The two PS genes encode proteins with similar function. Both PS genes encode the catalytic subunit of γ-secretase [33]. PS1 mutations are more severe and much more common than PS2 mutations [34]. AD-associated PS mutations can increase the Aβ42/Aβ40 ratio [35][36][37].

Double transgenic *App/Ps1* mouse from a cross line between *App* and *Ps1* gene is an acknowledged animal model. Double transgenic *App* $_{\text{K670N/M671L}}$/ *Ps1*$_{\text{M146L}}$ mice were generated by crossing *App* $_{\text{K670N/M671L}}$ single transgenic mice with *Ps1*$_{\text{M146L}}$ single transgenic mice. The mice exhibit β-amyloid (Aβ) deposition relatively early in the brain. The mutant *App/Ps1* mice express a human APP (K670N/M671L Swedish mutations) and

a mutant human PS1 (M146L) controlled by prion promoter elements (Figure 2). Excessive formation of Aβ plaques in the brain have been observed in *App/Ps1* transgenic mice. Studies consistently found memory loss and cognitive deficits in these mice. Short-term memory loss was detected at as early as age 3-month [38]. Previous studies including ours have shown that the spatial memory was impaired in 6-, 9- and 12-month-old *App/Ps1* mice when compared to wild-type [39][40][41]. These results suggest that these mice can provide researchers with various materials to investigate mechanisms of AD pathology and develop therapeutic agents. During my graduate study, I also used *App/Ps1* double transgenic mice in my research.

## 1.2 Oxidative stress in Alzheimer's disease

### 1.2.1 Oxidative stress

Oxygen metabolism is essential to cell life, but the process also forms partially reduced reactive oxygen species (ROS), a potential threat to cells [165][168]. The mitochondrial electron transport chain is responsible for most oxygen consumption and is a major source of ROS production [165][166]. Nicotinamide adenine dinucleotide phosphate oxidase- and cytochrome P450 oxygenases-catalyzed reaction also produce ROS [166]. A series of natural antioxidant defense systems normally eliminate ROS levels and limit tissue damage. ROS include superoxide ($O_2^-$), hydrogen peroxide ($H_2O_2$)

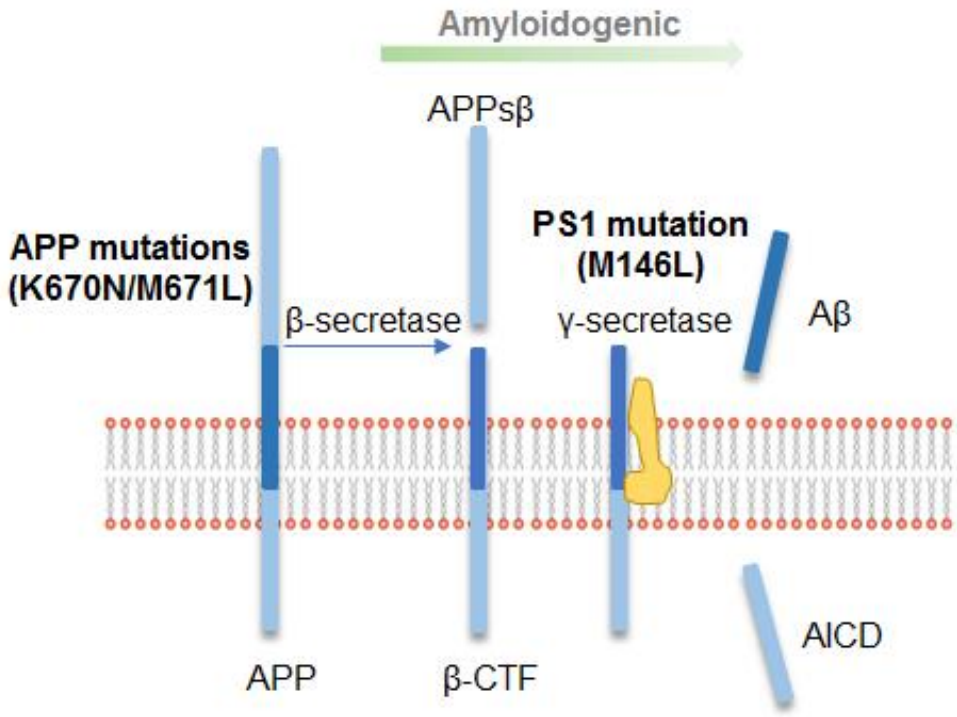


**Figure 2. Gene mutations of APP/PS1 animal model.** The APP/PS1 double transgenic mice overexpress a human APP (K670N/M671L) Swedish mutation and a mutant human PS1 mutation (M146L) on γ-secretase. Abbreviations: APP, Amyloid Precursor Protein; APPsβ, Soluble Amyloid Precursor Protein β; βCTF, β-secretase-cleaved C Terminal Fragment; AICD, Amyloid Precursor Protein Intracellular Domain.

and hydroxyl radical (HO•) [165][170]. As shown in Figure 3, $O_2^-$ is formed by the addition of one electron to oxygen ($O_2$) through a variety of mechanisms including enzymatic sources including mitochondrial electron transport chain, NADPH oxidase and cytochrome P450 oxygenases. $O_2^-$ radical is dismutated by superoxide dismutase (SOD) to $H_2O_2$, which is further converted to $H_2O$ by catalase. $H_2O_2$ also reacts with glutathione catalyzed by glutathione peroxidase and converts $H_2O_2$ to $H_2O$. HO• radical is generated in the presence of $H_2O_2$ and $Fe^{2+}$ by Fenton reaction [165][166][167]. Oxidative damage results from ROS overproduction that overrides the cellular antioxidative capacity to remove excess ROS [169][172]. Oxidative damage can also be caused by reactive nitrogen species (RNS) [170][171]. Nitric oxide (NO•) radical is produced during the oxygen-dependent conversion of L-arginine to L-citrulline, catalyzed by nitric oxide synthase (NOS). NO• radical can also react with $O_2^-$ to form peroxynitrite ($ONOO^-$). $ONOO^-$ is pH sensitive, at or below physiological pH it is protonated to form a peroxynitrous acid (ONOOH), which can undergo homolytic fission to generate hydroxyl (HO•) and nitrogen dioxide (NO2•) radicals [171].

The ROS/RNS are capable of damaging and modifying several types of macromolecules within the cell, including DNA, RNA, lipids, and proteins. These modifications may also generate potent reactive molecules [169][172].

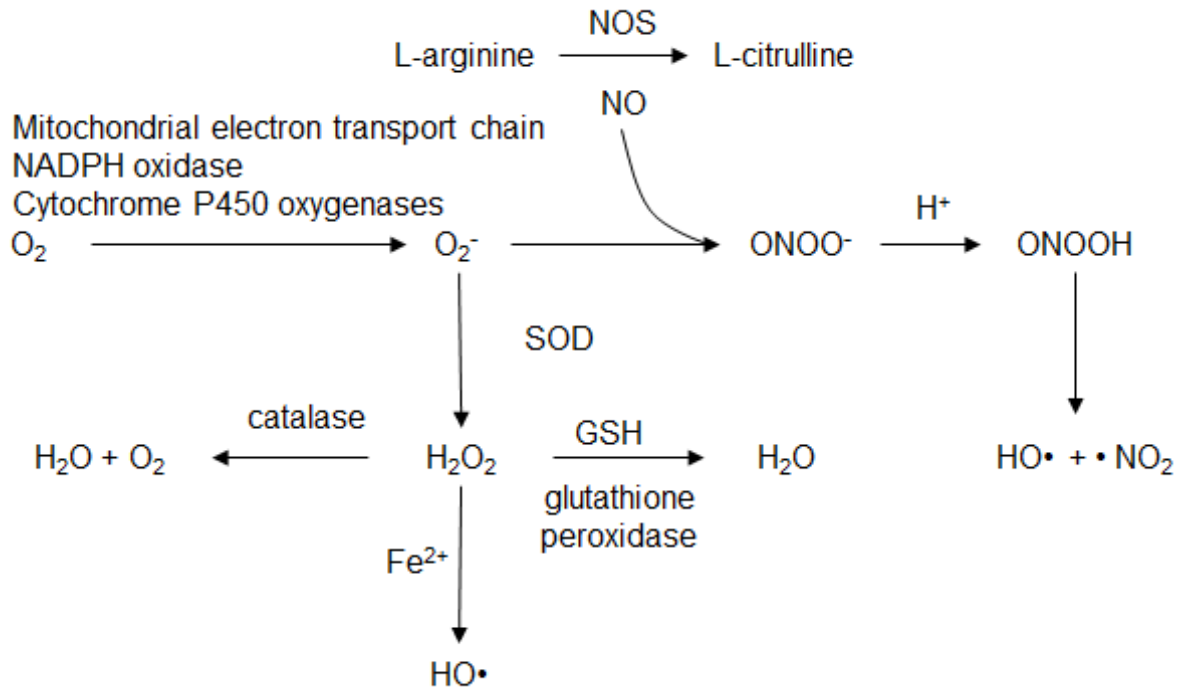


**Figure 3. Sources of ROS and RNS and some detoxification pathways.** $O_2^-$, superoxide; $H_2O_2$, hydrogen peroxide; HO•, hydroxyl radical; SOD, superoxide dismutase; NO, Nitric oxide; NOS, nitric oxide synthase; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; $NO_2$•, nitrogen dioxide

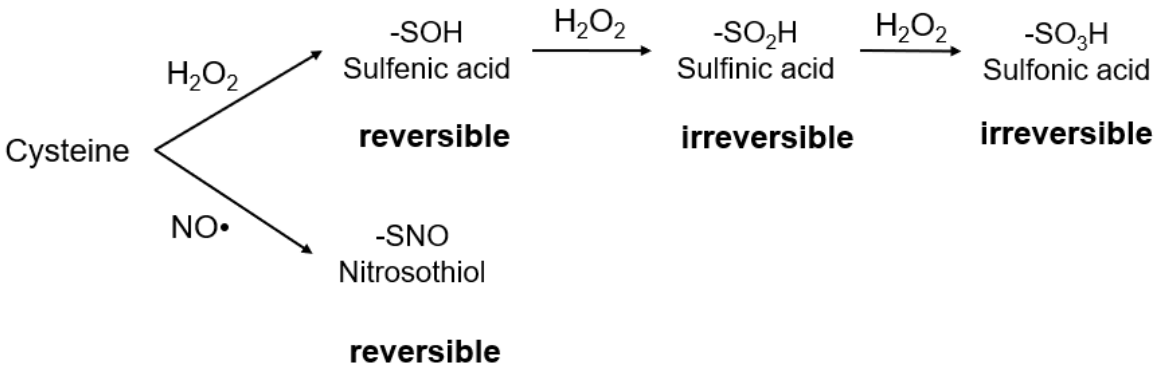


**Figure 4. Cysteine oxidative modifications.** $H_2O_2$ can oxidize Cysteine to generate reversible sulfenic acid (-SOH) (sulfenylation), further oxidized to irreversible sulfinic acid ($-SO_2H$) or sulfonic acid ($-SO_3H$). NO• radical can induce reversible nitrosylation of Cysteine to nitrosothiol (-SNO) (nitrosylation).

### 1.2.2 Oxidative stress in Alzheimer's disease

The brain is highly vulnerable to oxidative stress because it is very rich in easily peroxidizable fatty acids, has a high demand for oxygen and relatively lacks antioxidant systems. Mitochondrial dysfunction is major source for ROS overproduction. Studies have found that in the early stages of the disease, Aβ can enter the mitochondria, induce mitochondrial impairment, and increase ROS production and oxidative stress [42][43]. A significant amount of evidence has shown that oxidative stress is a critical pathological factor in AD. For example, studies showed higher levels of lipid peroxidation products in the hippocampus and pyriform cortex, and peripheral blood of patients with dementia of the Alzheimer type and mild cognitive impairment (MCI) [44][45]. A study also found that levels of 4-hydroxyhexenal (4-HNE), an end product of lipid peroxidation, were elevated in the hippocampus of the early stages of AD [46]. Other lipid peroxidation products F2-isoprostane and F4-neuroprostane were also found to be increased in frontal, parietal and occipital lobes in patients with MCI, and in patients at the late stage of AD [47]. Studies also reported high levels of protein carbonyls in frontal cortex of patients with MCI, mild AD, and AD when compared to health matched controls [48]. DNA oxidation marker, 8-hydroxy-2'-deoxyguanosine (8-OH-dG), was also significantly increased in hippocampus and CSF of patients with AD [49][50].

Oxidative damage has been found in brain of AD animal models. It was reported

that lipid peroxidation was increased, while antioxidants such as glutathione and vitamin E levels were decreased in brain of a triple-transgenic mouse model (3xTg-AD) harboring $PS1_{M146V}$, $APP_{Swe}$, and $tau_{P301L}$ transgenes [51]. Studies have also shown that levels of lipid peroxidation products were increased in plasma, urine and cerebral tissue of $APP_{Swe}$ transgenic mice models of AD [52]. Further, it has been found that protein oxidation was increased and SOD activity was impaired in brain of APP transgenic mice [53][54]. A dietary treatment with the potent polyphenolic antioxidant curcumin in $APP_{Swe}$ transgenic mice can decrease protein carbonyls and Aβ deposition [55].

Aβ has also been found to damage mitochondria and increase ROS production. Studies found that incubation with both Aβ25-35 and Aβ1-42 in isolated rat brain mitochondria can decrease the activity of mitochondrial complex IV [56][57]. Treatment with Aβ25-35 was also found to increase the levels of $H_2O_2$ and lipid peroxides in primary cultured rat cortical neurons and PC12 cell line [58]. Aβ1-42 has also been found to increase ROS production in cultured rat hippocampal neurons [59].

### 1.2.3 Protein cysteine oxidative modification in Alzheimer's disease

Proteins are critical in regulation of cellular structure and function. Oxidative damage to protein may result in various cellular functional changes. Cysteine (Cys) residues in protein play a significant role in many biological activities including enzyme

regulation, metal binding, gene transcription and others [60][61]. Cys thiols are very susceptible to attack by $H_2O_2$ and $NO^{\bullet}$ radicals. Both $H_2O_2$ and $NO^{\bullet}$ at pathological concentrations may target Cys and induce Cys oxidative modification, resulting in neuronal dysfunction and eventually neuronal cell death. As shown in Figure 4, $H_2O_2$ can oxidize Cys to generate reversible sulfenic acid (sulfenylation), subsequently either forming a disulfide bond with a nearby thiol or being further oxidized to irreversible sulfinic acid or sulfonic acid. NO• radical can also induce reversible nitrosylation of Cys [62].

A number of studies have shown that nitrosylation may contribute to AD pathophysiology. Previously our laboratory found that vesicular acetylcholine transporter, vesicular glutamate transporters and vesicular monoamine transporter 2 can be nitrosylated, and nitrosylation of these transporters can inhibit vesicular neurotransmitter uptake in mouse brain [63]. Further our laboratory has also found that not only total nitrosylated protein levels but also nitrosylation of vesicular acetylcholine and vesicular glutamate transporter were increased in brain of 9- and 12-month-old *App/Ps1* transgenic mice [41]. BACE1, insulin-degrading enzyme (IDE) and ApoE are three factors that regulate Aβ metabolism and aggregation. IDE is responsible for the clearance of various hormones and peptides, including insulin and Aβ [64]. ApoE represents a family of proteins enhancing the break-down of Aβ both within and between cells [65]. *In vitro*

studies have shown that all these three proteins can be S-nitrosylated in either cultured neurons or HEK-293 cell line [66][67][68]. Studies also found increased S-nitrosylation of BACE1, IDE, and ApoE in AD postmortem brains, suggesting that protein nitrosylation processes are important in Aβ deposition and AD development [66][68][69].

## 1.3 Thioredoxin antioxidant system

### 1.3.1 Structure and isoforms of thioredoxin

Thioredoxin as an oxidoreductase is highly conserved and ubiquitous in all cells. Trx was first identified as a hydrogen donor for ribonucleotide reductase in Escherichia coli [70]. There are two major isoforms of Trx, encoded by separate genes: cytosolic Trx (Trx1) and mitochondrial Trx (Trx2). Trx proteins in different organisms share a typical structure. It is composed of four α-helices and five β-sheets with a highly conserved active-site sequence, $Cys^{32}$-Gly-Pro-$Cys^{35}$. The thiol groups on cysteine residues play a critical role in the thiol-disulfide exchange reaction. Oxidation of a critical thiol group in cysteine will generally lead to a changed biological function. Besides the two cysteine residues, mammalian Trx1 contains three other critical cysteine residues at position -62, -69, and -73. These three Cys residues can undergo various post-translational modifications, such as thiol oxidation, glutathionylation, and S-nitrosylation [71].

### 1.3.2 Function of thioredoxin

Thioredoxin produces antioxidant activity, stimulates cell growth, inhibits apoptosis, activates numerous transcription factors and regulates the immune function. Thus, it is a promising therapeutic target in various diseases.

First, thioredoxin (Trx) as an oxidoreductase maintains a reduced environment in the cell, and renders protection against oxidative stress. Under oxidative stress conditions, Trx reduces cysteine oxidation modification. As shown in Figure 5, reduced Trx [Trx-$(SH)_2$] can reduce protein sulfenylation (-SOH) and disulfidation (Protein-$S_2$) by its general oxidoreductase activity, generating oxidized Trx (Trx-$S_2$). Trx-$(SH)_2$ can also reverse nitrosylation by reacting with nitrosothiols (-SNO), subsequently converting nitrosothiols to cysteine thiols and release nitroxyl ion (HNO). Further, Trx can reduce the oxidized form of Peroxiredoxin (Prx-$S_2$) to the reduced Prx-$(SH)_2$, and in turn scavenge $H_2O_2$ and other peroxides. Studies found that transient overexpression of Prx in cultured cells could eliminate the intracellular $H_2O_2$ generated in response to growth factors [72].

Second, Trx can inhibit the apoptotic process. Reduced Trx can bind to the N-terminal portion of apoptosis signal-regulating kinase 1 (ASK1), thereby inhibiting ASK1 activity [73]. Under the stimulation of oxidative stress and proinflammatory cytokines, ASK1 is dissociated from Trx and induces ASK1 phosphorylation,

subsequently resulting in JNK/p38 activation and cell apoptosis. It has been reported that association of Trx with ASK1 through a single cysteine (Trx Cys32 or Trx Cys35) is necessary and sufficient for Trx-induced ASK1 inhibition [74].

Third, Trx1 can also translocate into nucleus and interact with many transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1), thereby regulating gene expression. The transcription factors NF-κB and AP-1 have been implicated in the inducible expression of a variety of genes involved in responses to oxidative stress and cellular defense mechanisms. It was reported that Trx stimulates NF-κB DNA binding activity and promotes NF-κB-dependent gene expression in primary cultured human T lymphocytes [75][76]. It was also reported that overexpression of Trx in Hela cells can increase AP-1 DNA binding activity [77]. These results suggest that thioredoxin plays an important role in the regulation of transcriptional processes.

### 1.3.3 Regulation of thioredoxin

Reduced Trx is maintained by thioredoxin reductase (TrxR). As shown in Figure 5, in the presence of TrxR, the dithiol moieties of Trx are reduced by receiving electrons from NADPH. The selenocysteine (Sec) residue in thioredoxin reductase is conserved in mammalian species in a C-terminal-Gly-Cys-Sec-Gly motif, with the Sec residue being

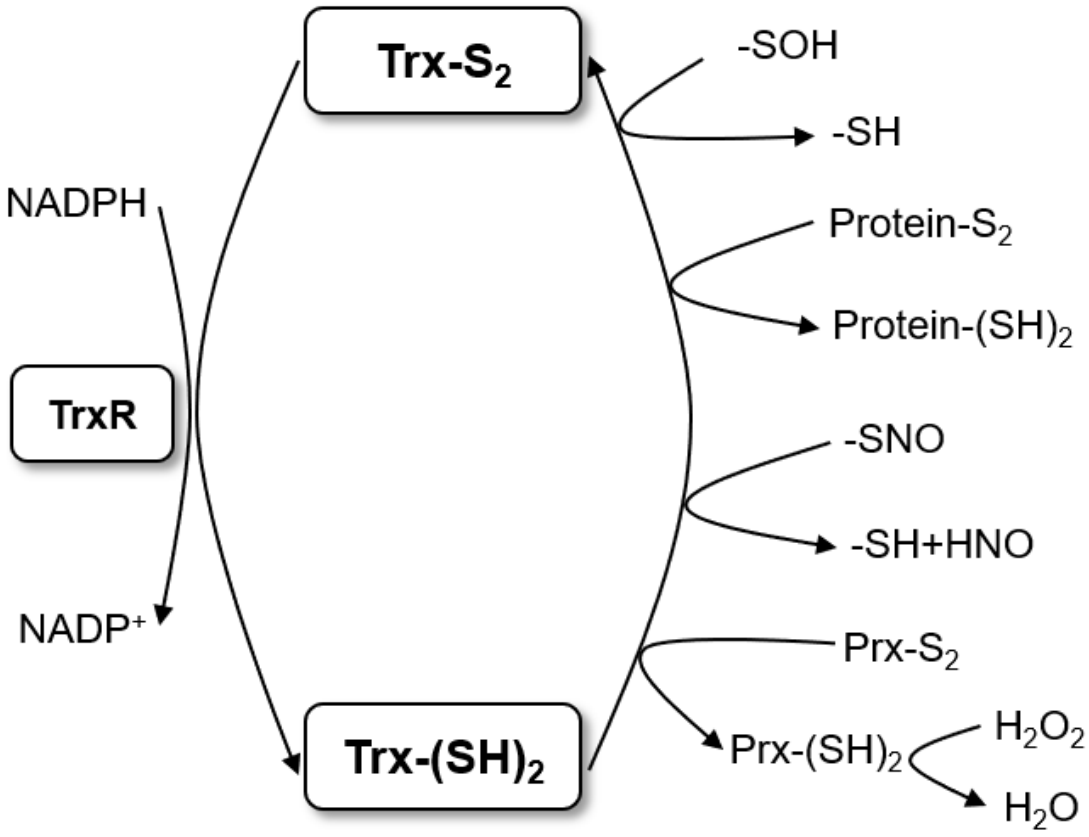


**Figure 5. Thioredoxin antioxidant system.** NADPH, nicotinamide adenine dinucleotide phosphate; $NADP^+$, oxidized form of NADPH; TrxR, thioredoxin reductase; Trx-$S_2$, oxidized form of thioredoxin; Trx-$(SH)_2$, reduced form of thioredoxin; -SOH, sulfenic acid (sulfenylation); -SH, thiol group; Protein-$S_2$, oxidized protein with disulfide bond (disulfidation); Protein-$(SH)_2$, protein with thiols; -SNO, nitrosothiols (nitrosylation); HNO, nitroxyl ion; Prx-$S_2$, oxidized form of peroxiredoxin; Prx-$(SH)_2$, reduced form of peroxiredoxin.

essential for catalytic activity [78][79].

Thioredoxin-interacting protein (Txnip) is an endogenous Trx inhibitor, interacting with the active center of Trx, thereby inhibiting Trx reducing activity. Txnip can form a disulfide bond between the Trx active site cysteine 32 and Txnip cysteine 247. The increase in Txnip-Trx complexes results in more oxidized proteins under oxidative stress.

As mentioned earlier, Trx1 can undergo post-translational modifications. For example, glutathionylation of Trx1 Cys73 was found to inhibit Trx1 activity under conditions of oxidative stress [80][137]. S-nitrosylation is a dynamic post-translational modification for the regulation of protein functions [81]. Studies have found that the activity of Trx1 increased upon S-nitrosylation. It was reported that S-nitrosylation of Trx Cys 69 enables Trx1 to scavenge ROS, preserve its redox regulatory activity and antiapoptotic function in endothelial cells [82].

Trx gene expression can be positively regulated by nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2). Nrf2 is a member of the cap ‘n’ collar (CNC) subfamily of basic region leucine zipper (bZip) transcription factors [83]. A primary emerging function of Nrf2 from studies over the past decade is its role in resistance to oxidative stress through resembling a common DNA sequence called antioxidant response element (ARE) [84]. Trx gene promoter region contains four functional ARE site. Nrf2 can directly induce Trx gene expression by binding to ARE [85]. Protein deglycase (DJ-1),

known as Parkinson disease protein 7, also showed protective effects on neurons against oxidative stress and cell death [86][87]. Studies showed that overexpression of DJ-1 can result in increased Nrf2 protein levels, promotes its translocation into the nucleus and enhances its recruitment onto the ARE site in the Trx1 promoter. In addition, DJ-1 knockdown in SH-SY5Y cells and DJ-1 knockout in mice significantly decreased Trx1 protein and mRNA levels [88].

### 1.3.4 Studies of thioredoxin antioxidant system in Alzheimer's disease

Studies have shown that Trx antioxidant system can be regulated in AD. Akterin *et al.* have reported that Trx1 protein levels were reduced in postmortem frontal cortex and hippocampal CA1 region of patients with AD [89]. Trx1 protein levels were also reduced in postmortem hippocampus and amygdala of patients with AD [90]. Increased TrxR levels were also found in postmortem amygdala and cerebellum of patients with AD [90]. In addition, Aβ1-42 was found to cause Trx1 oxidation in SH-SY5Y human neuroblastoma cells [89]. Treatment with recombinant Trx or TrxR inhibited Aβ-induced cytoxicity in primary cultured rat hippocampal cells [90]. These results indicate that dysregulation of Trx antioxidant systems could be essential events in AD pathogenesis.

Studies also found that knockout of *Trx* gene in mice is embryonically lethal in the homozygous mutants, but appears normal and viable in heterozygous mutants [138][139].

These results showed that Trx expression is essential for early differentiation of the mouse embryo. Previously *TrxR1* knockout mice were found significantly smaller and exhibited symptoms of ataxia and tremor. *TrxR1* knockout mice also showed reduced proliferation of the external granular layer and impaired fissure formation of the cerebellar cortex [140], indicating that *TrxR1* may play a critical role in the cerebellar morphogenesis. Txnip-deficient mice have fasting hypoglycemia and ketosis with a striking enhancement of glucose uptake by peripheral tissues [142][143]. Txnip is now known as a critical regulator of glucose metabolism [141][142]. So far, there is no study on Txnip gene deficiency in Alzheimer's disease.

## 1.4 Summary

Aβ plaques are the primary pathological characteristics found in AD patients [1][2][3]. AD animal models that elevate Aβ show AD-like behaviors such as memory loss and aggressive behaviors [35][38]. Many studies have consistently shown that ROS production, lipid peroxidation and protein oxidation were increased in brain of AD patients, brain of AD animal models and Aβ-treated cells [48][50][53]. Since oxidative stress significantly contributes to neurodegeneration and inflammation processes, oxidative stress-caused damage plays an important role in AD development [54][55]. Trx is a small redox protein that plays a vital role in the regulation of oxidative protein

cysteine modification [71][72]. A Trx reduced state is maintained by TrxR, and Txnip is an endogenous inhibitor for Trx. Trx system is a crucial antioxidant system for protection against oxidative stress and maintenance of the cellular redox balance [78][93]. Deficiency of Trx system may play an important role in AD development.

### 1.5 Overall objectives of this project

In order to understand the role of Trx antioxidant system in AD development, I analyzed Trx and Txnip in brain of *App/Ps1* transgenic mice and Aβ-treated neuronal cells. My research objectives are:

1. To determine the regulation of thioredoxin system in the brain of *App/Ps1* mice
2. To determine the effect of Aβ on Trx and Txnip in cultured neuronal cells.
3. To determine the role of thioredoxin system in Aβ-induced oxidative protein modification

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Aβ peptide preparation

Preparation of Aβ1-42 oligomers was performed as previously described [91]. Aβ1-42 peptide (abcam, US) was dissolved in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) (sigma-aldrich, Canada) at a concentration of 0.5 mM and incubated for 1 hour at room temperature for complete solubilization. The solution was dried under a fume hood overnight. The peptide film can be sealed the next day and stored at -20°C. The peptide film was suspended in dimethyl sulfoxide (DMSO) to a final concentration of 5mM. Then DMSO solution of Aβ1-42 was diluted in phosphate buffered saline (PBS) to a concentration of 100μM (2% DMSO in the stock solution), incubated at 4°C for 24 hours. Finally, the concentration of Aβ1-42 was determined by Bradford protein assay. The results have shown that the concentration of Aβ1-42 in the stock solution is approximately 50 μM.

Acetyl-Amyloid β 25-35 (Aβ25-35) was purchased from Sigma-Aldrich Canada. Lyophilized Aβ25-35 was dissolved in PBS to obtain a stock solution at 1mM and stored at -20°C until use. Aβ25-35 was directly added to cell culture medium at 20μM concentration.

## 2.2 Cell culture

HT22 mouse hippocampal cell line was generously provided by the Salk Institute (La Jolla, CA, USA). HT22 cells were maintained in Dulbecco's Modified Eagle Media (DMEM) (Life Technologies Inc, Burlington, ON, Canada) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum, and cultured at 37°C under 5% $CO_2$.

Primary cultured mouse cerebral cortical neurons were performed as previously described [92]. Embryonic fetuses at day 17-18 were removed from the uterus and the cerebral cortex was dissected by Dr. Michael Jackson's lab. After I received cerebral cortex, I removed meninges and blood vessels, digested tissue with 0.25% trypsin for 15 minutes at 37°C, followed by DNase (0.7mg/ml) digestion for another 15 minutes at 37°C. Then digestion procedure was terminated with 10% fetal bovine serum. After precipitation, supernatant was discarded. Then tissue was dissociated mechanically by pipetting and resuspended in 1ml fresh neurobasal medium (Life Technologies Inc) with 1X GlutaMax (Life Technologies Inc), 1X GS21 supplement (Sigma-Aldrich Canada), 1% of penicillin/streptomycin. Cells were seeded onto poly-D-lysine-coated (50ug/ml) cell culture plates at a density of $3\times10^5$ cells/ml. Medium was half changed twice per week. Cells were maintained in culture at 37°C under 5% $CO_2$ for 10 days before Aβ treatment.

## 2.3 Tissue processing and protein isolation

***Protein isolation from mouse brain tissue:*** Mouse hippocampus and frontal cortex were dissected and homogenized at 10:1 (ml/g) ice-cold lysis buffer containing 20 mM HEPES (pH 7.5), 250 mM NaCl, 20% glycerol, 30 mM $MgCl_2$, 0.5 mM EDTA, 0.1mM ethylene glycol tetraacetic acid (EGTA), 1% nonidet P40 and 1× protease inhibitor cocktail (Thermo Scientific, Marietta, OH, USA). The homogenized tissues were kept on ice for 1 h and then centrifuged at 10,000X g for 15 min at 4°C. The supernatants were then collected as protein extract. Bradford protein assay was used to determine protein concentrations (Bradford, 1976).

***Protein isolation from cultured cells:*** Cells were washed twice, scraped with ice-cold PBS and then collected by centrifuge at 1000X g for 5 min at 4°C. Cell lysis procedure was the same with the procedure used for mouse brain tissue as described above. The cell lysate were kept on ice for 1 h and then centrifuged at 10,000X g for 15 min at 4 °C. The supernatant was collected as protein extract. Protein concentrations were determined by the Bradford protein assay (Bradford, 1976).

## 2.4 Immunoblotting analysis

Protein samples were mixed with a loading buffer containing 100mM Tris-HCl (pH 6.8), 200 mM dithiothreitol (DTT), 4% sodium dodecyl sulfate (SDS), 0.2%

bromophenol blue and 20% glycerol. Protein samples were loaded to electrophoresis in 12% SDS polyacrylamide gels for 1 hour at 120V. Then proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA) for 2 hours at 220 mA on ice. Membrane blots were first blocked with 5% milk in Tris-buffered saline (TBS) containing 10mM Tris-HCl (pH 7.4) and 0.1% Tween-20 at room temperature for 1h. The membrane was incubated overnight at 4°C with primary antibody of rabbit monoclonal Txnip (1:2000 dilution, Abcam Inc., Toronto, ON, Canada) or rabbit monoclonal Trx1 (1:7000 dilution, Cell Signaling Technology, Danvers, MA, USA). Then the membrane was further incubated with secondary antibody of goat anti-rabbit conjugated to horseradish peroxidase (1:5000, Abcam, Eugene, Oregon, USA) for 1 h at room temperature. The membranes were developed using enhanced chemiluminescence reagents (PerkinElmer, Waltham, MA, USA), and then images were captured by the ChemiDoc MP System (Bio-Rad, Dreieich, Germany). Band signal intensity was quantified by measuring the density of the band using Image Lab (Bio-Rad).

## 2.5 Protein sulfenylation and nitrosylation

***Dimedone conjugation assay for detection of cysteine sulfenylated proteins [93]:***

Sulfenylated thiols was first reacted with dimedone to generate irreversible dimedone-derivatized proteins that then were subjected to SDS-PAGE gel, transferred to

membranes and analyzed by western blot with anti-dimedone antibody. Cells were washed with PBS and digested with 0.25% trypsin. Then the reaction was terminated with Dulbecco's modified Eagle medium containing 5% dimedone and 10% fetal bovine serum. After cells were centrifuged at 1000g, cell pellet was collected and lysed on ice for 30 min with a lysate buffer containing dimedone and catalase (2mM dimedone, 3 mM citric acid, 0.1% Triton-X and 12 mM sodium phosphate dibasic, 10mM iodoacetamide, 10mM N-ethylmaleimide, 200 units/ml catalase, 10mM EDTA and 0.01% SDS). The lysates were centrifuged at 10,000g for 10 min and the supernatants were collected. The protein was quantified by Bradford protein assay (Bradford, 1976). Fifteen μg of protein was used to analyze sulfenylated proteins using immunoblotting analysis with anti-cysteine sulfenic acid antibody at 1:3000 dilution (Millipore Canada Ltd, Etobicoke, ON, Canada).

***Biotin-switch assay for detection of cysteine nitrosylated proteins [41]:***

First, unmodified thiols in cysteine were blocked with a thiol-specific methylthiolating agent methyl methanethiosulfonate (MMTS) (MMTS cannot block oxidative modified thiols). Then nitrosylated thiols in cysteine were reduced back to free thiols by ascorbate. The newly formed free thiols were labeled with N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propionamide (biotin-HPDP). Biotinylated protein (nitrosylated protein) was resolved by SDS-PAGE, transferred to membrane and detected by anti-biotin

antibody. One hundred μg of protein in 50 μl was incubated with 200μl blocking solution (40mM MMTS, 2.5% SDS, 250 mM HEPES pH 7.7, 1 mM EDTA and 0.1 mM neocuproine) at 50°C for 40 min, and then added with 1 ml cold 99% acetone and further incubated at -20 °C for 40 minutes for precipitation. The reaction mixture was centrifuged at 13,000x g, at 4°C for 15 min. After supernatant was removed, the pellet was resuspend with 15μl HENS buffer (250mM HEPES pH 7.7, 0.1mM neocuproine and 1mM EDTA, 1% SDS), and added with 250 μl of 50 mM ascorbate and 4mM biotin-HPDP, and incubated at room temperature for 1 h. Fifteen μl from each sample was used to analyze nitrosylated proteins using immunoblotting analysis with polyclonal anti-biotin antibody at 1:2000 dilution (Sigma, St. Louis, MO, USA).

**2.6 Generation of Txnip knockout cells**

Txnip single guide RNAs (sgRNAs) or scrambled sgRNAs (as control), and CRISPR/Cas9 All-in-One lentivector pLenti-U6-sgRNA-SFFV-Cas9-2A-Puro were purchased from ABM Inc (Richmond, BC, Canada). The plasmids were packaged by lentivirus and transfected into HT22 cells. Then stably transfected HT22 cells were selected using the antibiotic Puromycin. Lentiviral packaging, gene transfection and stable transfection were performed by Dr. Eftekhar Eftekharpour's laboratory. Briefly HEK293T cells were transfected with Cas9-sgRNA constructs and packaging plasmids

(GAG, VSVG, REV and TAT) [93][94]. Then the media containing lentiviral particles was collected after 48 hour HEK293T cell transfection. The lentiviral particles were used to further transfected into HT22 cells using polybrene as transfection agent. Stably transfected HT22 cells were selected by passaging in media containing 4µg/ml Puromycin. Single clones from scramble and Txnip knockout cells were selected and propagated for further experiments.

### 2.7 Statistical analysis

IBM SPSS 24.0 software (IBM, Armonk, New York, USA) was used to perform statistical analysis. All results were expressed as mean ± standard error of the mean (SEM). Significant differences among means were analyzed by one-way analysis of variance (ANOVA) with Tukey post hoc comparisons. Student's t-tests were used for statistical analysis of two groups. A p value of less than 0.05 was regarded as statistically significant.

# CHAPTER 3: RESULTS

## 3.1 To determine if Trx is downregulated and Txnip is upregulated in brain of *App/Ps1* mice

### 3.1.1 10μg of protein is appropriate for immunoblotting analysis of Trx and Txnip protein levels

Protein at amount of 2.5, 5, 10, 20 and 40 μg from HT22 cell lysates were loaded in 12% of SDS-PAGE gel. Trx and Txnip protein levels were measured using immunoblotting analysis. As shown in Figure 6A and 6B, 12 KD band was identified as Trx, and 55 KD band was identified as Txnip. Intensity was increased when protein amounts were increased. Based on these results, I used 10 μg for my research.

### 3.1.2 Txnip protein levels are increased in the frontal cortex and hippocampus of 9 and 12-month-old *App/Ps1* mice

Proteins were isolated from frontal cortex and hippocampus of 3, 6, 9 and 12-month-old *App/Ps1* and wild type mice, and Trx and Txnip protein levels were measured by immunoblotting analysis. As shown in Figure 7A and 7B, we found that Trx protein levels were not changed in frontal cortex of 6, 9 and 12-month-old *App/Ps1* mice and hippocampus of 3, 6, 9 and 12-month-old *App/Ps1* mice, but Trx protein levels were increased in frontal cortex of 3-month-old *App/Ps1* mice when compared to controls.

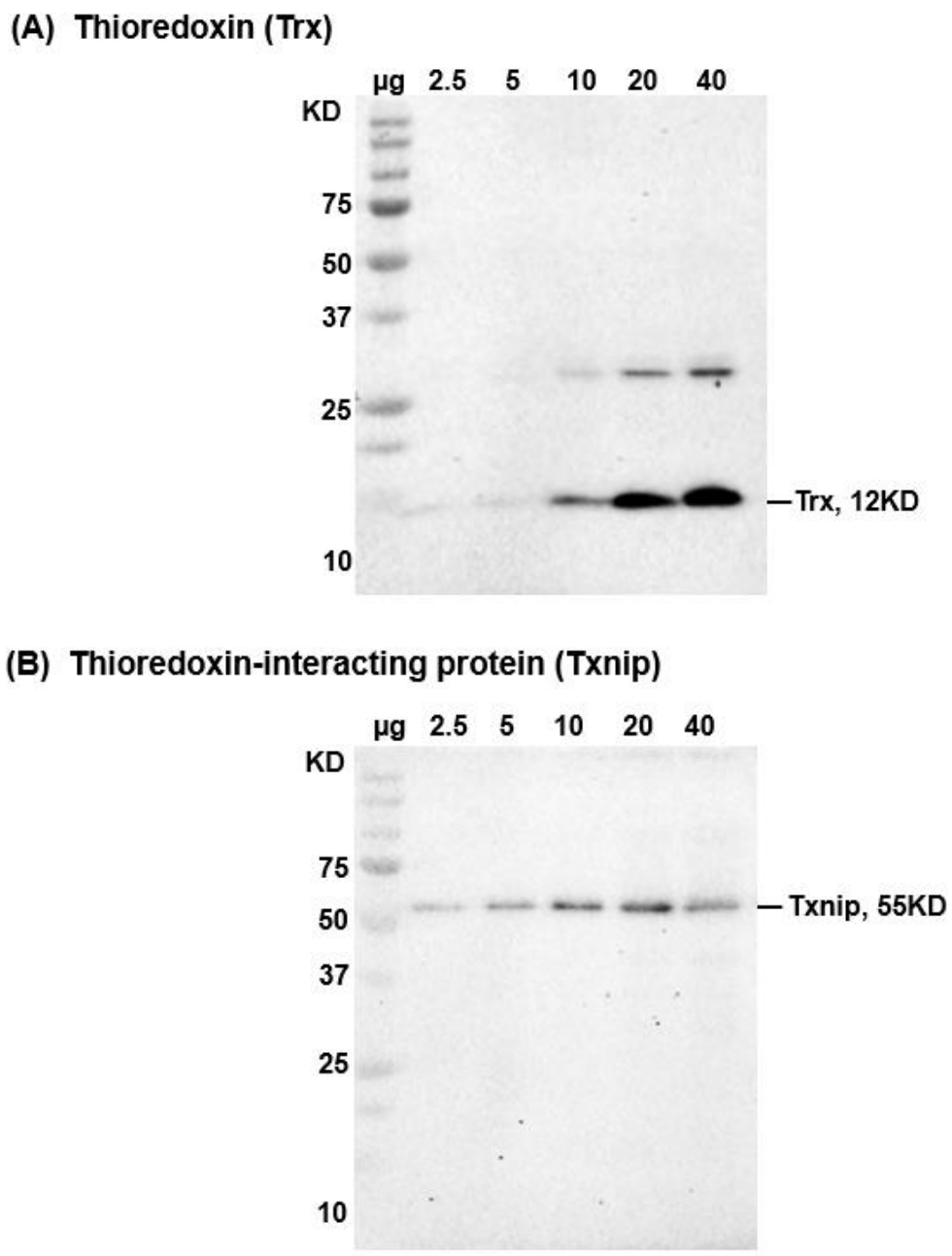


**Figure 6.Whole blot of Thioredoxin (A) and Thioredoxin-interacting protein (B).** Protein at amount of 2.5, 5, 10, 20 and 40 μg from HT22 cell lysates were loaded in 12% of SDS-PAGE gel. Immunoblotting analysis was performed using primary antibody of rabbit monoclonal Trx1 (1:7000 dilution, Cell Signaling Technology, Danvers, MA, USA) (A) or rabbit monoclonal Txnip (1:2000 dilution, Abcam Inc., Toronto, ON, Canada) (B).

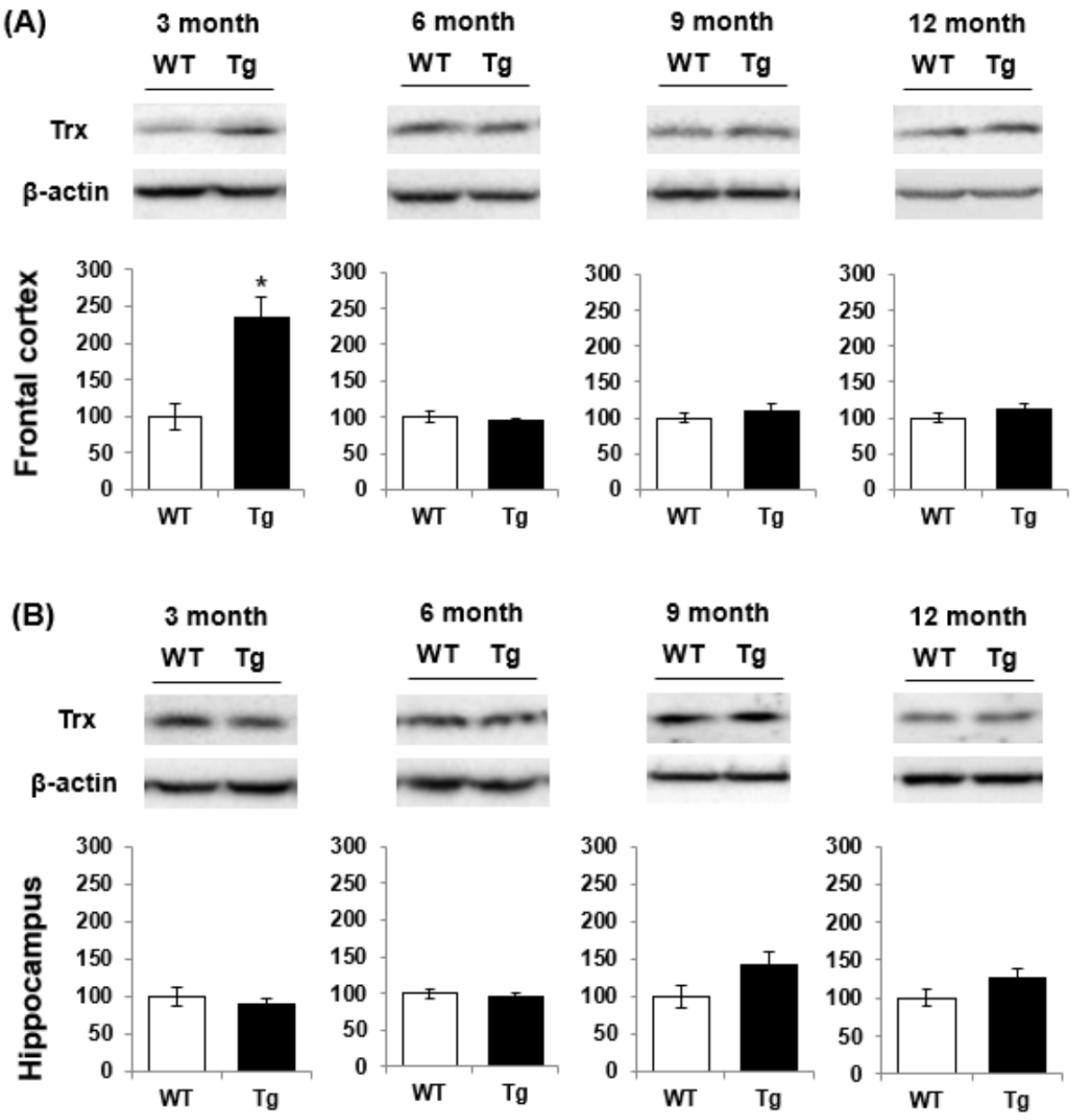


**Figure 7. Trx protein levels in frontal cortex (A) and hippocampus (B) of 3-, 6-, 9-, 12-month-old wild type (WT) mice and APP/PS1 transgenic mice (Tg).** Data are displayed as mean ± SEM, 3 month: N=11 for wild type, N=10 for APP/PS1; 6 month: N=12 for wild type, N=6 for APP/PS1; 9 month: N=10 for wild type, N=9 for APP/PS1; 12 month: N=9 for wild type, N=5 for APP/PS1.

However, we found that although Txnip protein levels were not significantly changed in frontal cortex of 3 and 6-month-old *App/Ps1* mice, Txnip protein levels were significantly increased in 9 and 12-month-old *App/Ps1* mice when compared to their wild type littermates (Figure 8A). In the hippocampus region (Figure 8B), Txnip protein levels were also significantly increased in 9 and 12-month-old *App/Ps1* mice, but not in 3 and 6-month-old *App/Ps1* mice when compared with wild type mice.

### 3.1.3 Sulfenylated protein levels are increased in frontal cortex and hippocampus of 9 and 12-month-old *App/Ps1* mice

Txnip can inhibit Trx activity, subsequently promoting cysteine oxidative modification such as nitrosylation and sulfenylation. Previously we have found that total nitrosylated protein levels were significantly increased in frontal cortex and hippocampus of 9, 12-month-old *App/Ps1* mice [41]. In the present study, I also investigated total sulfenylated protein levels in frontal cortex and hippocampus of 9 and 12-month-old *App/Ps1* mice. Sulfenylated protein levels were measured by dimedone conjugation assay. As shown in Figure 9A and 9B, although total sulfenylated protein levels was not significantly changed in frontal cortex of 9-month-old *App/Ps1* mice when compared to wild type mice, sulfenylated protein levels were significantly increased in frontal cortex of 12-month-old *App/Ps1* mice and in hippocampus of 9 and 12-month-old *App/Ps1* mice

when compared with wild type mice.

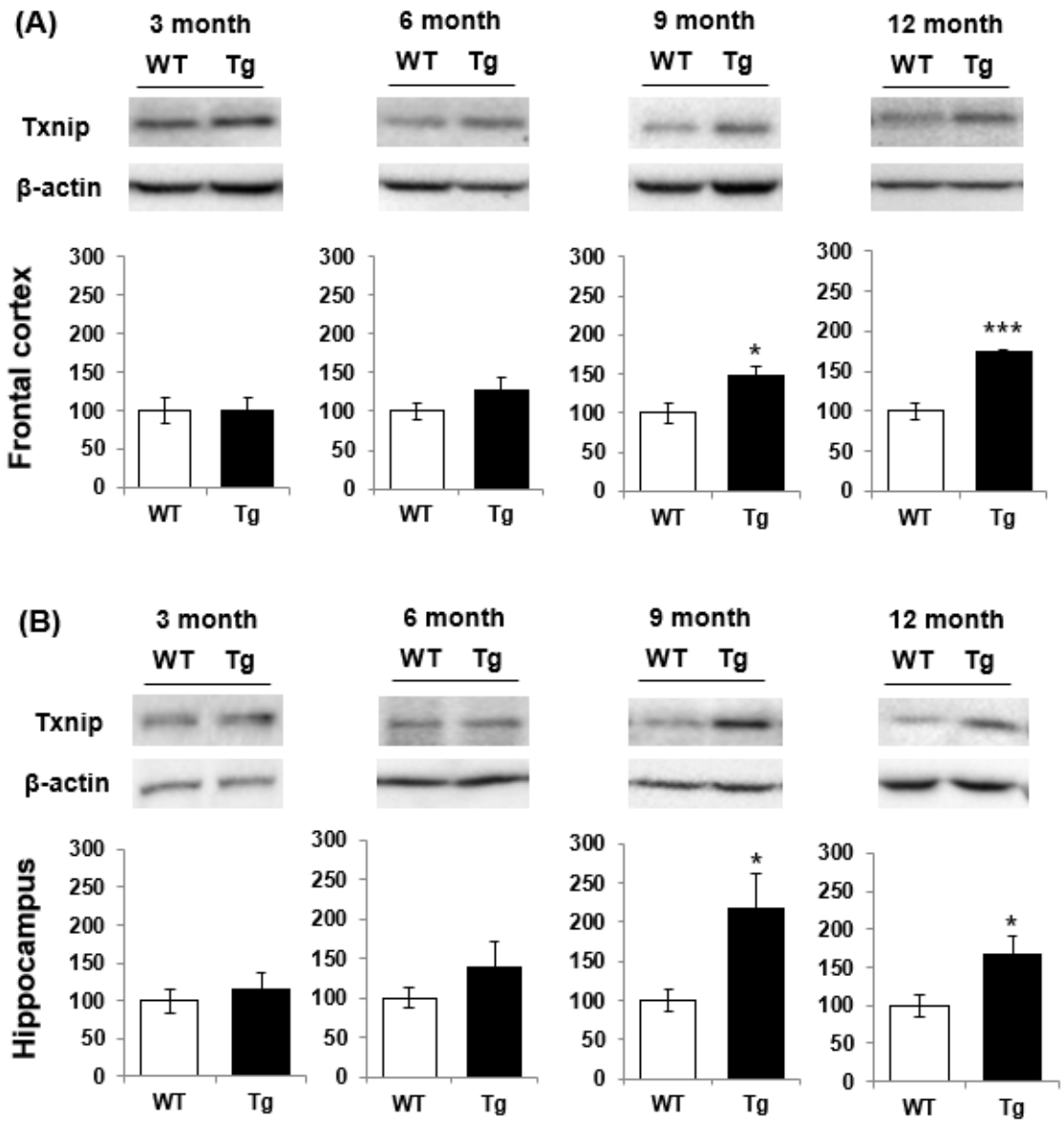


**Figure 8.Txnip protein levels in frontal cortex (A) and hippocampus (B) of 3-, 6-, 9-, 12-month-old wild type (WT) mice and APP/PS1 transgenic mice (Tg).** Data are displayed as mean ± SEM, 3 month: N=11 for wild type, N=10 for APP/PS1; 6 month: N=12 for wild type, N=6 for APP/PS1; 9 month: N=10 for wild type, N=9 for APP/PS1; 12 month: N=9 for wild type, N=5 for APP/PS1. * indicates $p < 0.05$, * * * indicates $p < 0.001$ by student's t test.

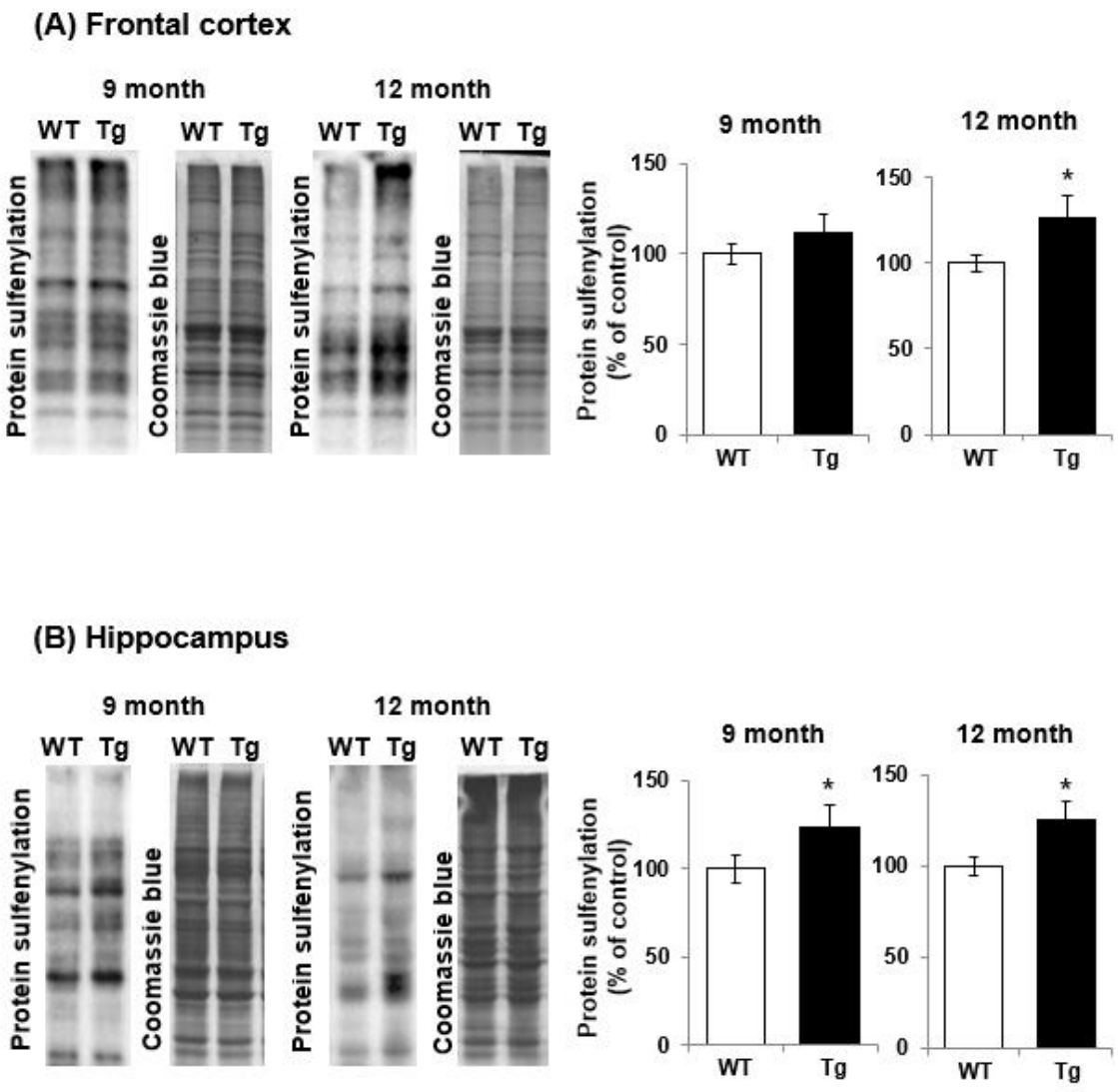


**Figure 9.Sulfenylated protein levels in frontal cortex (A) and hippocampus (B) of 9-, 12-month-old wild type (WT) mice and APP/PS1 transgenic mice (Tg).** Data are displayed as mean ± SEM. 9 month: N=10 for wild type, N=9 for APP/PS1; 12 month: N=9 for wild type, N=5 for APP/PS1. * indicates $p < 0.05$, * * * indicates $p < 0.001$ by student's t test.

### 3.2 To determine the effect of Aβ on Trx and Txnip protein levels in cultured neuronal cells

#### 3.2.1 Aβ25-35 increases Txnip protein levels in HT22 mouse hippocampal cells and primary cultured cerebral cortical neurons

Aβ production is significantly increased in brain of *App/Ps1* transgenic mice. To determine if increased Txnip protein levels in brain of *App/Ps1* mice are directly caused by Aβ, I measured the effect of Aβ on Txnip protein levels in cultured neuronal cells. Aβ25-35 is an 11-amino acid fragment of the Aβ peptide. Though not found in the brain of AD patients, Aβ25-35 can produce similar toxic effects to those caused by Aβ1-42 such as neuronal death, protein oxidation and lipid peroxidation (Yankner et al., 1989; Pike et al., 1995; Yatin et al., 1999). Therefore, I measured the effects of both Aβ25-35 and Aβ1-42 on Txnip protein levels.

First, I analyzed the effect of Aβ25-35 on Trx and Txnip protein levels in cultured HT22 cell line. HT22 cells were treated with Aβ25-35 at 20μM for 1, 3 and 5 days. Protein levels were analyzed by immunoblotting analysis. As shown in Figure 10B, I found that treatment with 20μM Aβ25-35 for 1 day, 3 days and 5 days all significantly increased Txnip protein levels in HT22 cells. It is interesting that treatment with 20μM Aβ25-35 for 1 day, 3 days and 5 days also significantly increased Trx protein levels in HT22 cells (Figure 10A). Further I verified the effect of Aβ25-35 on Txnip and Trx

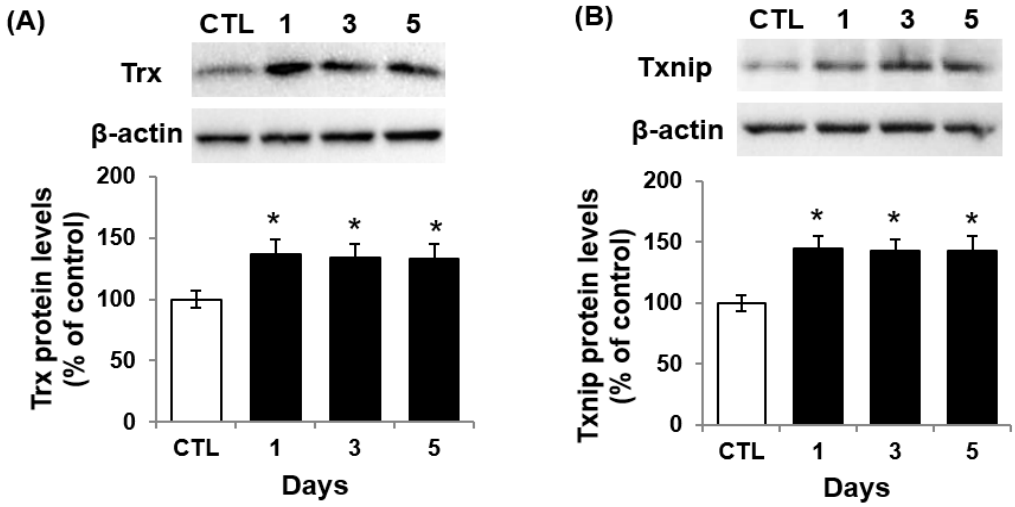


**Figure 10.The effect of Aβ25-35 on Trx (A) and Txnip (B) protein levels in HT22 cells.** HT22 cells were treated with vehicle (CTL) or Aβ25-35 at 20μM for 1 day, 3 days, and 5 days, respectively. Protein levels of Trx and Txnip were measured with each antibody using immunoblotting analysis. β-actin was used as a normalization standard. Results are shown as mean ± SEM (N=6). *Indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

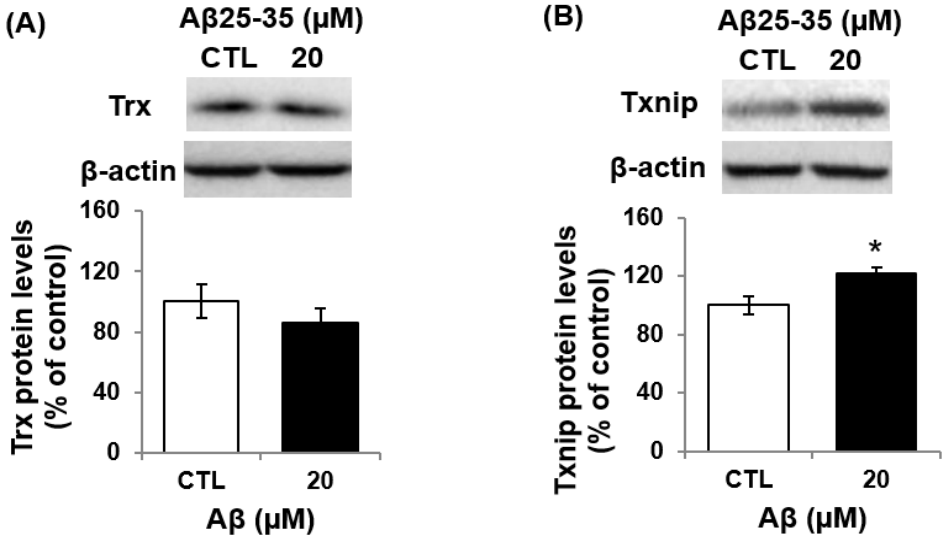


**Figure 11.The effect of Aβ25-35 on Trx (A) and Txnip (B) protein levels in cerebral cortical neurons.** Neurons were treated with vehicle (CTL) or Aβ25-35 at 20μM for 1 day. Protein levels of Trx and Txnip were measured with each antibody using immunoblotting analysis. β-actin was used as a normalization standard. Results are shown as mean ± SEM (N=6). *Indicates $p<0.05$ when compared to controls determined by Student's t-test.

protein levels in primary cultured mouse cerebral cortical neurons. Cultured cortical neurons were treated with Aβ25-35 at 20μM for 1 day. I found that although treatment with Aβ25-35 had no effect on Trx protein levels (Figure 11A), this treatment significantly increased Txnip protein levels (Figure 11B).

### 3.2.2 Aβ1-42 increases Txnip protein levels in HT22 mouse hippocampal cells and primary cultured cerebral cortical neurons

Because Aβ1-42 peptide is naturally present in the human brain and the oligomeric form is the most toxic, I measured the effect of Aβ1-42 oligomers on Trx and Txnip protein levels in HT22 cells. Cells were treated with Aβ1-42 at 1 and 3μM for 1 day. Protein levels were analyzed by immunoblotting analysis. As shown in Figure 12A, I found that Aβ1-42 at 1 and 3μM for 1 day significantly increased Trx protein levels. I also found that Aβ1-42 at 1 and 3μM for 1 day significantly increased Txnip protein levels (Figure 12B). It has been reported that Aβ1-42 at lower concentrations can produce protective effects in neurons (Puzzo et al., 2008). Therefore, I also analyzed the effect of Aβ1-42 at lower concentrations on Trx and Txnip protein levels. Cells were treated with Aβ1-42 at 0.2 nM and 200 nM for 1 day. As shown in Figure 13A, although Aβ1-42 at 0.2 nM and 200 nM for 1 day significantly increased Trx protein levels, these treatments had no effect on Txnip protein levels (Figure 13B).

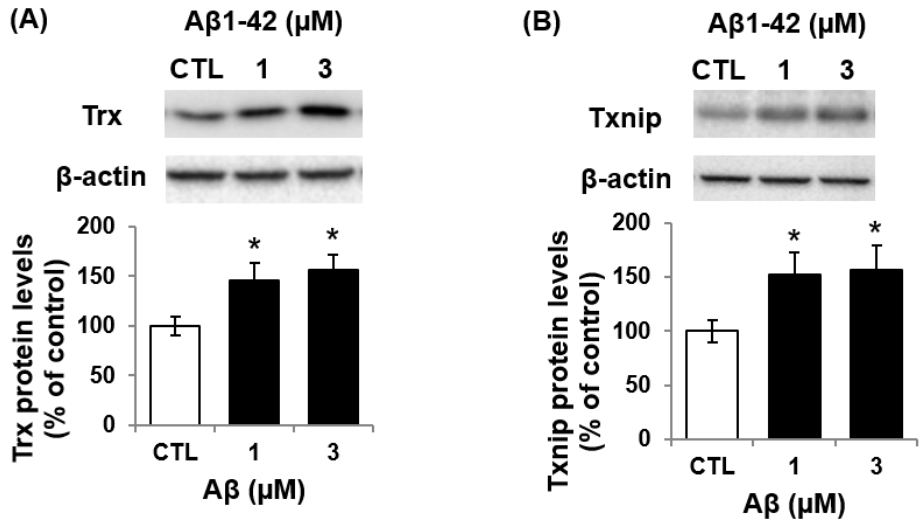


**Figure 12.The effect of Aβ1-42 on Trx (A) and Txnip (B) protein levels in HT22 cells.** HT22 Cells were treated with vehicle (Ctl) or Aβ1-42 at 1 and 3 μM for 24 hours. Protein levels of Trx and Txnip were measured with each antibody using immunoblotting analysis. β-actin was used as a normalization standard. Results are shown as mean ± SEM (N=4). *Indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

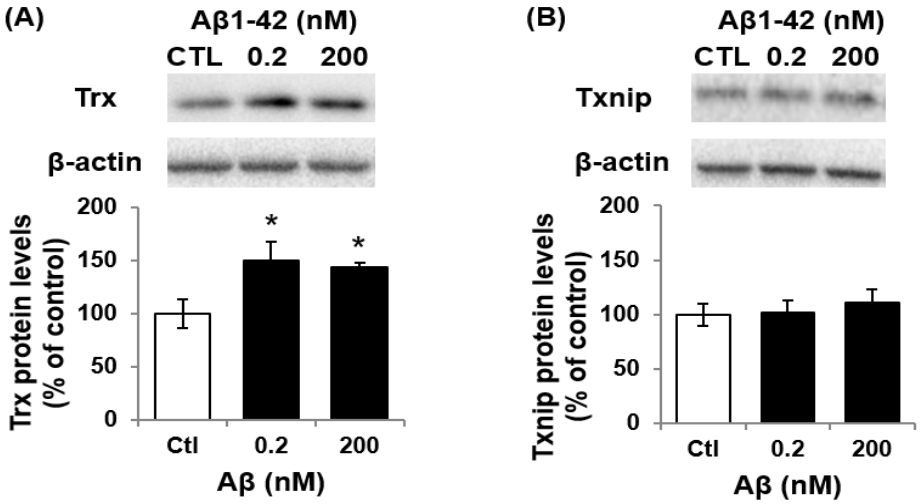


**Figure 13. The effect of Aβ1-42 at lower concentrations on Trx (A) and Txnip (B) protein levels in HT22 cells.** HT22 Cells were treated with vehicle (Ctl) or Aβ1-42 at 0.2 and 200 nM for 1 day. Protein levels of Trx and Txnip were measured with each antibody using immunoblotting analysis. β-actin was used as a normalization standard. Results are shown as mean ± SEM (N=4). *Indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

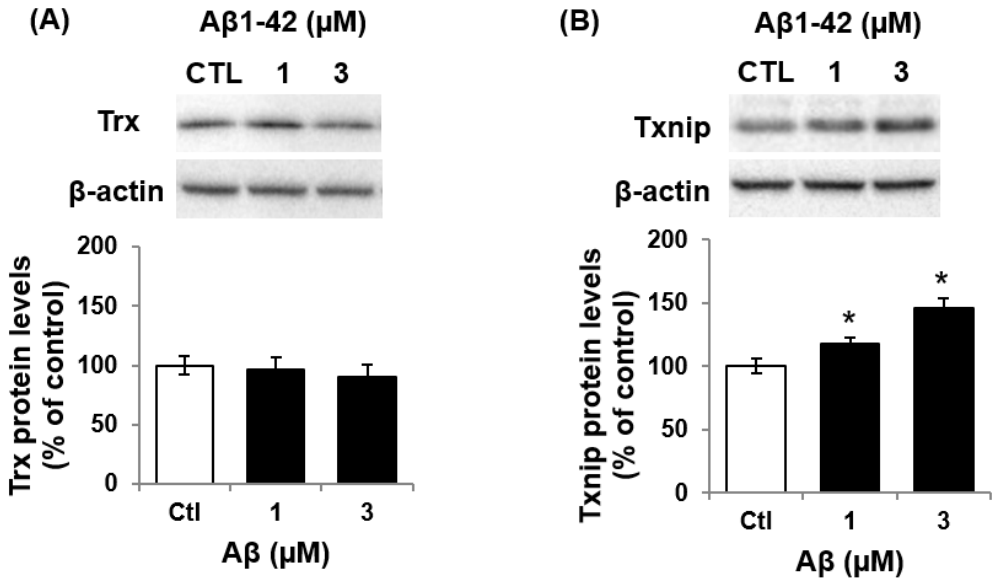


**Figure 14. The effect of Aβ1-42 on Trx (A) and Txnip (B) protein levels in mouse cerebral cortical neurons.** Neurons were treated with vehicle (CTL) or Aβ1-42 at 1 and 3 μM for 1 day. Protein levels of Trx and Txnip were measured with each antibody using immunoblotting analysis. β-actin was used as a normalization standard. Results are shown as mean ± SEM (N=6). *Indicates $p<0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post-hoc analysis.

I also further verified the effect of Aβ1-42 on Trx and Txnip protein levels in primary cultured mouse cerebral cortical neurons. Cultured neurons were treated with Aβ1-42 at 1 and 3μM for 1 day. As shown in Figure 14A, although treatment with 1 or 3μM Aβ1-42 had no effect on Trx protein levels, these treatments significantly increased Txnip protein levels (Figure 14B).

### 3.3 To determine the effect of Aβ on protein sulfenylation and nitrosylation

#### 3.3.1 Aβ25-35 and Aβ1-42 increase protein cysteine sulfenylation and nitrosylation in HT22 mouse hippocampal cells

$H_2O_2$ and NO radical can attack cysteine thiol groups and cause cysteine sulfenylation and nitrosylation, which are the part of oxidative damage to protein. Because Trx can reverse protein cysteine sulfenylation and nitrosylation, while Txnip can inhibit Trx activity, enhancing cysteine sulfenylation and nitrosylation, Aβ-increased Txnip may further promote cysteine sulfenylation and nitrosylation. Therefore, I measured the effect of Aβ on protein sulfenylation and nitrosylation in HT22 mouse hippocampal cells. As shown in Figure 15, we found that Aβ25-35 at 10 and 20 μM significantly increased sulfenylated protein levels. I also found that Aβ25-35 at 5, 10 and 20 μM significantly increased nitrosylated protein levels (Figure 16).

Next, we measured the effect of Aβ1-42 on cysteine sulfenylation and nitrosylation. $H_2O_2$ can attack protein cysteine residues and induce cysteine sulfenylation. As shown in Figure 17, we found that both $H_2O_2$ at 300 μM (as the positive control) and Aβ1-42 at 3 μM significantly increased sulfenylated protein levels. S-nitrosoglutathione (GSNO) is a NO donor that can induce cysteine nitrosylation. As shown in Figure 18, we also found that both GSNO at 200 μM (as the positive control) and Aβ1-42 at 3 μM significantly increased nitrosylated protein levels.

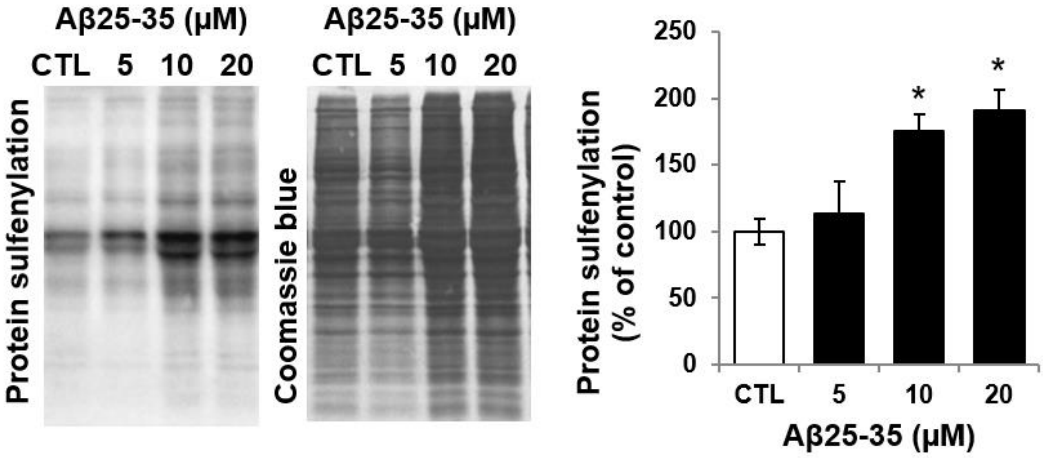


**Figure 15. The effect of Aβ25-35 on protein sulfenylation in HT22 cells.** Cells were treated with vehicle (CTL) or Aβ25-35 at 5, 10 and 20 μM for 1 day. Sulfenylated protein levels were measured by dimedone conjugation assay, followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 6). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

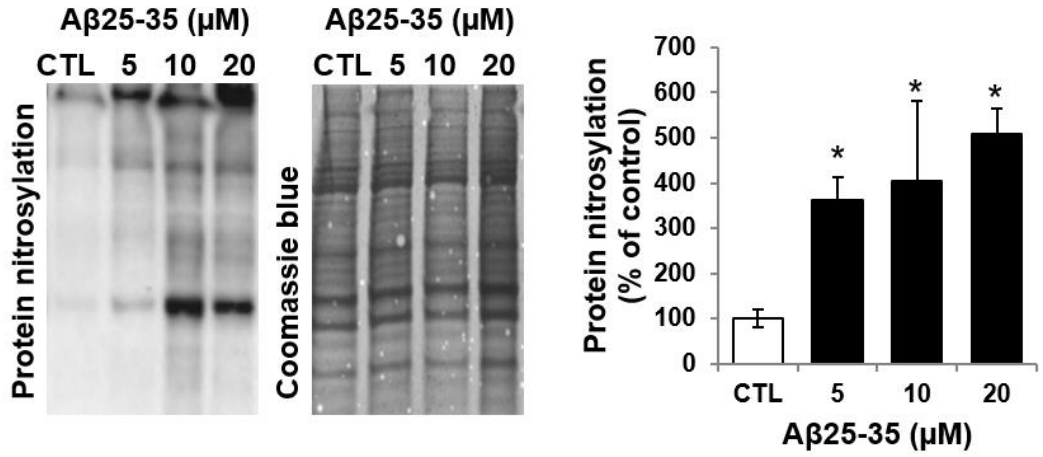


**Figure 16. The effect of Aβ25-35 on protein nitrosylation in HT22 cells.** Cells were treated with vehicle (CTL) or Aβ25-35 at 5, 10 and 20 μM for 1 day. Nitrosylated protein was measured by biotin-switch method, followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 6). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

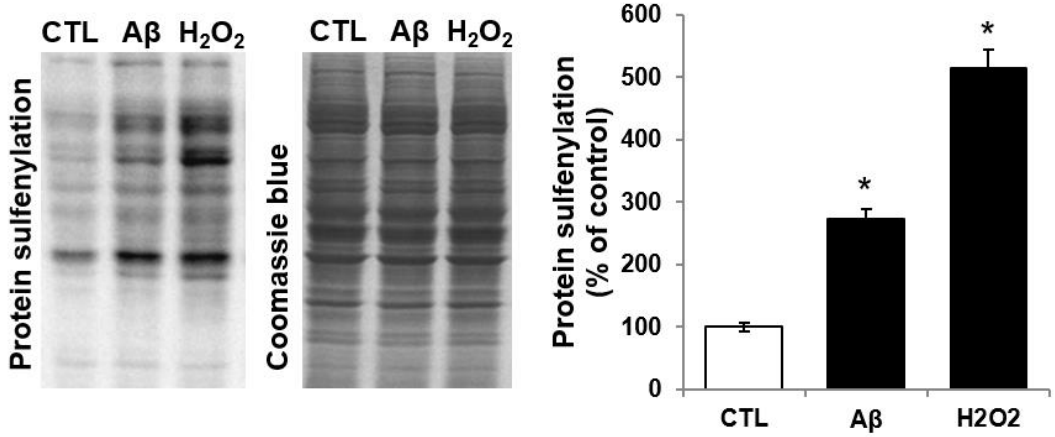


**Figure 17. The effect of Aβ1-42 on protein sulfenylation in HT22 cells.** Cells were treated with vehicle (CTL) and Aβ1-42 at 3μM for 1 day. Cells were also treated with $H_2O_2$ at 300μM for 30 minutes as the positive control. Sulfenylated protein levels were measured by dimedone conjugation assay, followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 5). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

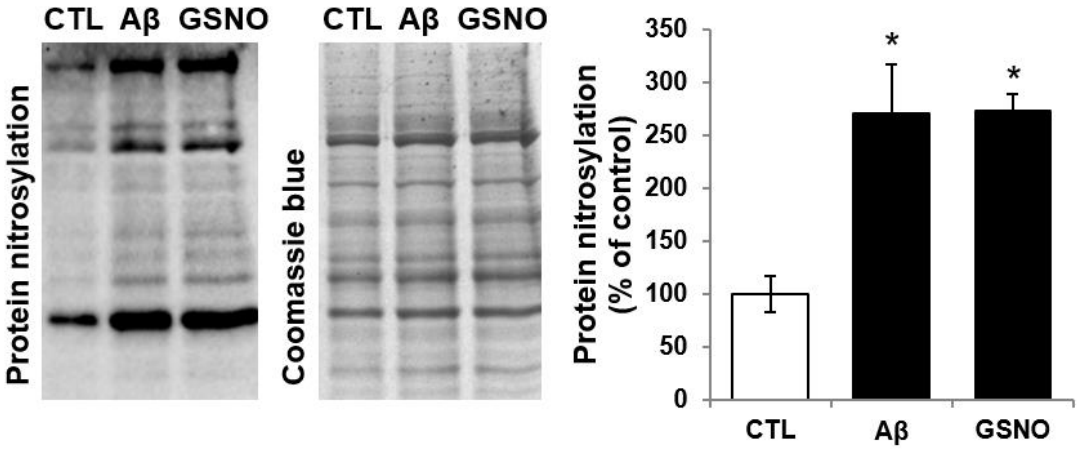


**Figure 18. The effect of Aβ1-42 on protein nitrosylation in HT22 cells. Cells were treated with vehicle (CTL) and Aβ1-42 at 3μM for 1 day.** Cells were also treated with GSNO at 200μM for 30 minutes as the positive control. Nitrosylated protein was measured by biotin-switch method, followed by immunoblotting analysis. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 6). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

### 3.3.2 Knocking out Txnip reduces Aβ-increased protein cysteine sulfenylation and nitrosylation in HT22 mouse hippocampal cells

To determine if Txnip mediates Aβ-increased protein cysteine sulfenylation and nitrosylation, I analyzed the effect of Txnip knockout on Aβ-increased protein cysteine sulfenylation and nitrosylation in HT22 cells. Txnip gene was knocked out by the clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 technology. As shown in Figure 19A, the Txnip single guide RNA (sgRNA) is a short synthetic RNA composed of a scaffold sequence necessary for Cas9-binding, and a 20 nucleotide spacer sequence that is complementary to the specific Txnip gene. The Cas9 protein and the sgRNA can form a ribonucleoprotein complex, and Cas9 will cleave the targeted Txnip DNA sequence. In the present study, lentivirus-packaged Txnip sgRNA (AGAACGAGATGGTGATCATG) and CRISPR/Cas9 lentivector were stably transfected into HT22 cells. Scrambled sgRNA was used as control. Then Txnip protein levels were measured using immunoblotting analysis. As shown in Figure 19B, Txnip protein levels were significantly knocked out in cells transfected with Txnip sgRNAs compared with cells transfected with scramble sequence. This result suggests that Txnip sgRNA totally blocked Txnip gene expression.

Next, we measured the effect of Aβ on protein sulfenylation and nitrosylation in Txnip knockout HT22 cells. Cells were treated with Aβ1-42 at 3μM for 1 day. I found

that treatment with Aβ significantly increased sulfenylated protein levels in cells transfected with scrambled sequence, but this treatment had no effect on sulfenylated protein levels in cells transfected with Txnip sgRNAs (Figure 20). As shown in Figure 21, we also found that treatment with 3μM Aβ1-42 significantly increased nitrosylated protein levels in cells transfected with scrambled sgRNAs. I also found that although treatment with 3μM Aβ1-42 significantly increased nitrosylated protein levels in cells transfected with Txnip sgRNAs, magnitude of increase by Aβ1-42 in cells transfected with Txnip sgRNAs was lower than in transfected with scrambled sgRNAs.

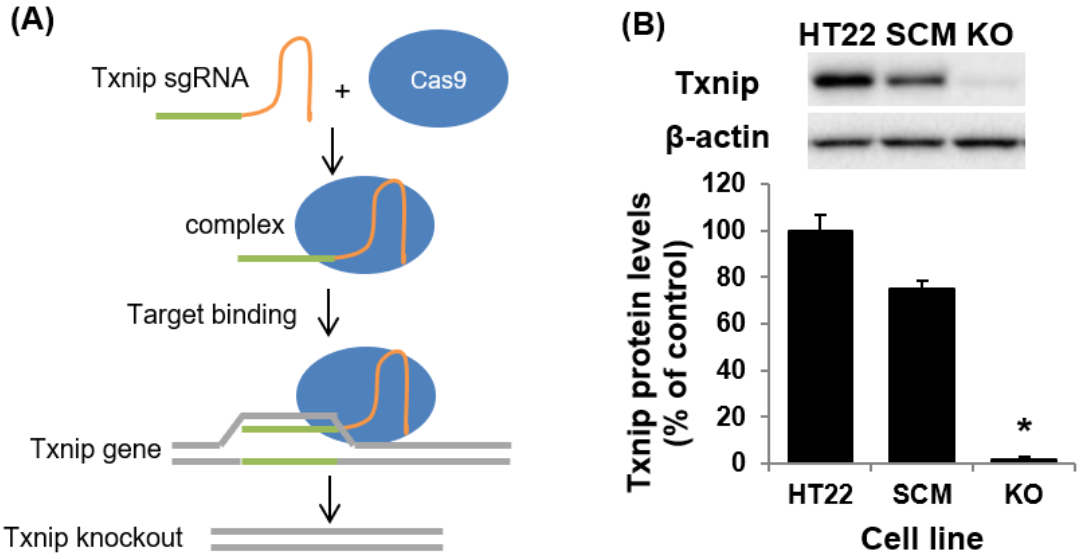


**Figure 19. The mechanism of CRISPR/Cas9 system. (B) Txnip protein levels were measured in HT22 cell line, cells transfected with scrambled (SCM) sequence and cells transfected with Txnip sgRNAs (KO).** Txnip protein levels were measured by immunoblotting analysis. Data are displayed as mean ± SEM (N = 5). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by student's t test.

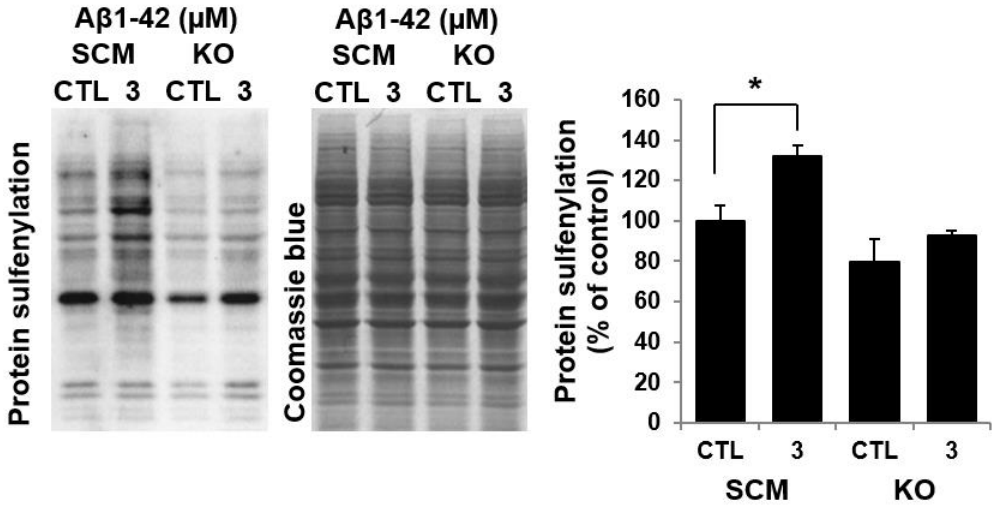


**Figure 20. Effect of Txnip sgRNAs on Aβ-increased protein sulfenylation.** HT22 cells transfected with Txnip sgRNAs or scrambled sgRNAs were treated with vehicle (CTL) or Aβ1-42 for 24h. Sulfenylated protein was measured by dimedone conjugation method. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 5). * indicates $p < 0.05$ when compared to controls determined by one-way ANOVA followed by Tukey's post hoc test.

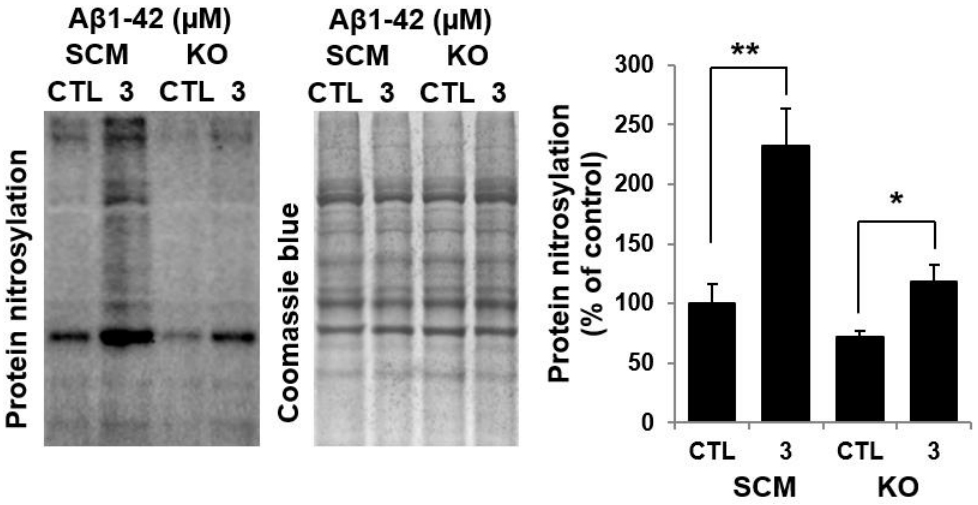


**Figure 21. Effect of Txnip sgRNAs on Aβ-increased protein nitrosylation.** HT22 cells transfected with Txnip sgRNAs or scrambled sgRNAs were treated with vehicle (CTL) or Aβ1-42 for 24h. Nitrosylated protein was measured by biotin switch method. Band signal intensity in whole lane was quantitated by densitometry. The membrane was striped and stained with coomassie blue used as loading control. Data are displayed as mean ± SEM (N = 6). * indicates $p < 0.05$ when compared to controls determined by Student's t test.

# CHAPTER 4: DISCUSSION

## 4.1 Aβ increases Txnip expression

In the present study, we found that Txnip protein levels were significantly increased in hippocampus and frontal cortex of 9- and 12-month-old *App/Ps1* double transgenic mice when compared to controls. Previously it was found that Txnip protein levels were also increased in the hippocampus of 4-month-old 5XFAD mice when compared to controls [95]. 5XFAD transgenic mice were generated by combining five AD-related mutations [APP K670N/M671L (Swedish) + I716V (Florida) + V717I (London) and PS1 M146L+ L286V]. These mice develop cerebral amyloid plaques at 2 months of age and develop massive Aβ42 burdens, neuron loss and memory impairment [96]. Since *App/Ps1* and 5XFAD mice exhibit increased Aβ levels in brain, these results suggest that Txnip can be upregulated by Aβ. Indeed, we further found that treatment with Aβ increased Txnip protein levels in HT22 mouse hippocampal cells and primary cultured cerebral cortical neurons.

It is of interest that we also found that Trx protein levels were significantly increased only in the frontal cortex of 3-month-old *App/Ps1* mice compared with controls. I also found that Aβ at 1 and 3 μM increased Trx protein levels in HT22 cells. Because Aβ at these concentrations has been reported to cause oxidative stress [97][98]. Aβ-increased Trx may result from increased oxidative damage, which compensatively

upregulates Trx and temporarily counteracts oxidative stress. For example, previously it was reported that overexpression of Trx1 protected against Aβ-induced toxicity in SH-SY5Y cells [99]. Txnip persistently increased by Aβ may overcome compensatively upregulated Trx and eventually cause oxidative damage. However, Trx protein levels are not changed by Aβ in primary cultured mouse cerebral cortical neurons. It is possible that the Trx antioxidant defense system is weaker in mouse cerebral cortical cells than in HT22 cell line.

Txnip produces Trx dependent and independent functions. As shown in Figure 22, Txnip Cys247 can interact with Trx active center cysteine-32 in cytosol and mitochondria. Txnip-Trx binding inhibits Trx activity [100][101]. Trx can reverse protein cysteine sulfenylation and nitrosylation, and reduce oxidized Prx, facilitating Prx-induced scavenge of $H_2O_2$ and other peroxides [75][145]. Therefore, binding of Txnip to Trx promotes oxidative stress. Under basal conditions, Trx binds to ASK1, prevents activation of c-Jun N-terminal kinase (JNK) and P38 mitogen-activated kinase (P38), and inhibits ASK1/JNK/P38-mediated stress and inflammatory signaling [73] [146]. Binding of Txnip to Trx releases ASK1, induces ASK1 phosphorylation and activates ASK1/JNK/P38 signaling [73][74]. In addition, Txnip plays an important role in activating nod-like receptor protein 3 (NLRP3) inflammasome through directly binding to NLRP3 [149]. The NLRP3 inflammasome is a multiprotein complex that controls the activation of

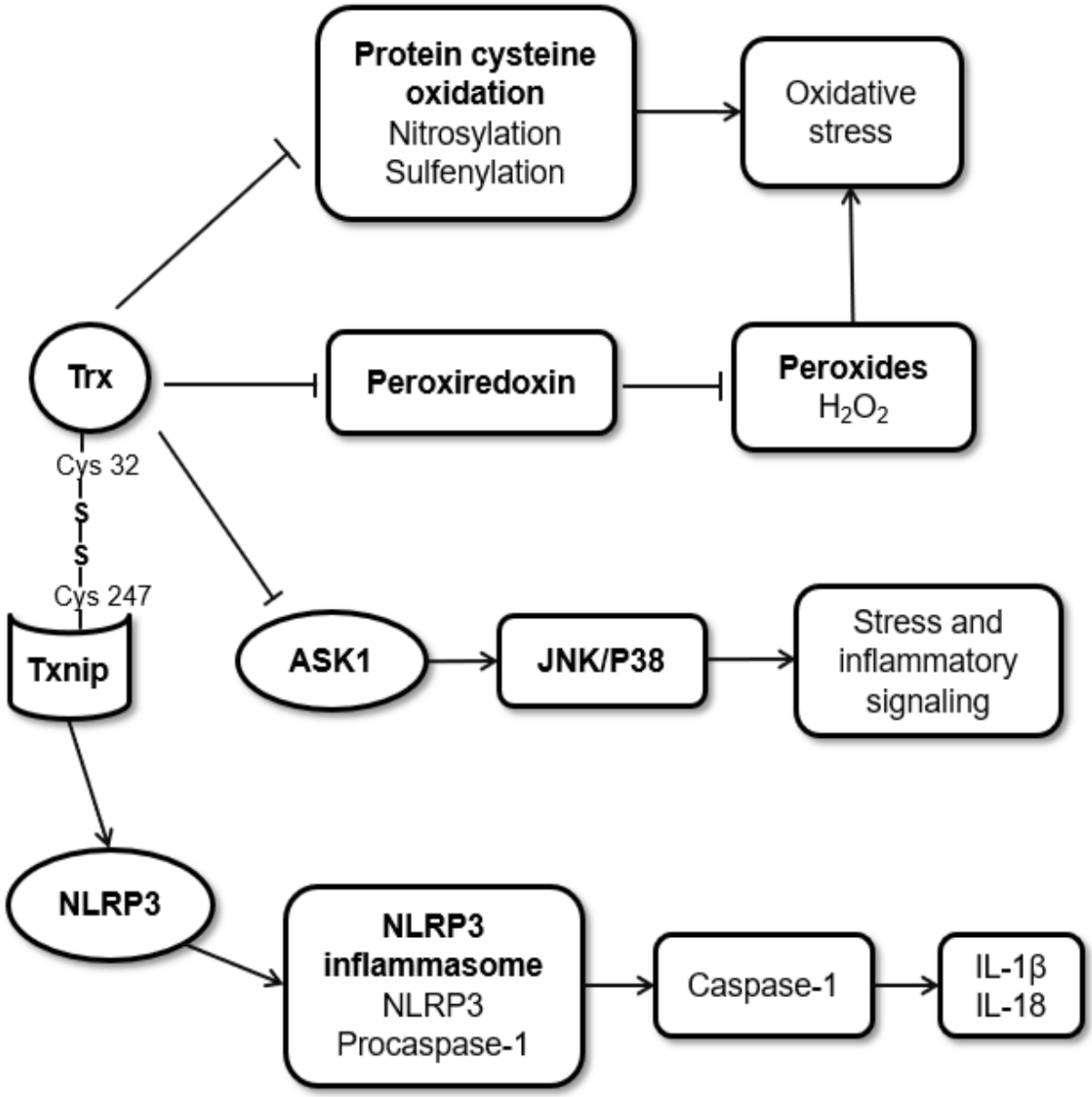


**Figure 22. Effected targets of thioredoxin-interacting protein (Txnip).** Txnip, Trx-interacting protein; Trx, thioredoxin; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; P38, P38 mitrogen-activated kinase; NLRP3, nod-like receptor protein 3; IL, interleukin; ⟞, suppress; ⟶, activate

caspase-1 in the innate immune system, resulting in maturation of IL-1β and IL-18 [102][103]. Binding of Txnip to NLRP3 facilitates forming NLRP3 inflammasome, subsequently cleaving procaspase-1 into the active caspase-1 that converts pro-interleukin (IL)-1β and pro-IL-18 into IL-1β and IL-18, which accelerate proinflammatory responses [125][130]. Because Txnip levels are increased in brain of *App/Ps1* mice and in Aβ-treated cultured cells, our findings suggest that Aβ may target Txnip, resulting in oxidative stress and inflammation.

**4.2 Aβ increases protein cysteine oxidative modification**

Aβ may increase Txnip protein levels and further cause protein cysteine oxidative modification, which impairs neuronal functions. Previously we reported that nitrosylated protein levels were significantly increased in hippocampus and frontal cortex of 9- and 12-month-old *App/Ps1* transgenic mice [41]. In the present study, we also found that sulfenylated protein levels were increased in frontal cortex and hippocampus of 9- and 12-month-old *App/Ps1* mice. I also found that treatment with Aβ increased sulfenylated and nitrosylated protein levels in HT22 cells [41]. Our findings suggest that Aβ-upregulated Txnip may facilitate protein cysteine sulfenylation and nitrosylation processes.

To further understand the role of Txnip in Aβ-induced protein cysteine oxidative

modification, we analyzed the effect of knocking out Txnip on Aβ-induced protein nitrosylation and sulfenylation. I found that knocking out Txnip totally reversed Aβ-increased sulfenylated protein levels and partially reduced Aβ-increased nitrosylated protein levels. Our findings indicate that Aβ treatment may increase Txnip levels, inhibiting Trx activity and promote protein cysteine sulfenylation and nitrosylation.

Protein cysteine residues are involved in many protein functions including enzymatic catalysis, homeostasis and metal binding [60]. Cysteine is highly vulnerable to attack by reactive oxygen/nitrogen species, resulting in cysteine oxidative modification. Oxidative modification of cysteine residues may interrupt many cellular processes, which further contributes to pathophysiological development of AD.

Cholinergic deficits and glutamate-induced excitotoxicity play important roles in AD development. Previously our laboratory found that vesicular acetylcholine transporter and vesicular glutamate transporter can be nitrosylated and nitrosylation of these transporters can inhibit their neurotransmitter uptake functioning [41][63]. We further found that nitrosylation of vesicular acetylcholine transporter and vesicular glutamate transporter was increased in hippocampus and frontal cortex of *App/Ps1* transgenic mice [41]. These results suggest that the nitrosylation process may contribute to impairment of cholinergic and glutamatergic neurotransmission in AD. Pin1 is a peptidyl-prolyl cis/trans isomerase and isomerizes only phospho-serine/ threonine/proline motifs. Pin1-induced

conformational changes affect various protein functions such as phosphorylation status, protein interaction, and protein stability [104][105]. It was found that the sulfenylation of Cys113 on Pin1 was significantly elevated in the postmortem AD human brain and in APP transgenic mice. The oxidation of Cys113 induced Pin1 inactivation and mislocalization [106]. This suggests that Pin1 oxidation-induced disruption of protein phosphorylation, protein interaction and protein stability are important in AD pathology. These findings, together with our results, also suggest that Aβ-increased Txnip may promote protein cysteine oxidative modification, which may further damage vesicular transporters and various enzymes, and impair neuroplasticity.

Prx scavenges $H_2O_2$ and other peroxides. A Prx reducing state is maintained by Trx. Aβ may increase Txnip levels, inhibit Trx reducing activity for peroxiredoxin, and subsequently decrease scavenging of $H_2O_2$ and peroxides. Previously it has been reported that $H_2O_2$ levels were increased in the brains of 6 and 10-month-old *App/Ps1* mice [107]. It has been also found that intracerebroventricular delivery of Aβ1-40 or Aβ25-35 into rats increased levels of ROS and lipid peroxides in the cerebral cortex and hippocampus [108]. Further in vitro studies found that treatment with Aβ25-35 for 24 hours increased levels $H_2O_2$ and lipid peroxides in both cultured B12 cell line and primary cultured hippocampal neuron [58]. Aβ1-40 was also found to increase levels of lipid peroxides in cultured rat hippocampal H19-7 neuronal cell line [109]. These studies further support

that Aβ-increased Txnip may further enhance ROS production and lipid peroxidation.

### 4.3 Aβ activates ASK1/JNK/p38 apoptotic signaling

Aβ may upregulate Txnip and further inhibit Trx activity, activating ASK1/JNK/p38 apoptotic signaling. ASK1 is a protein kinase of the mitogen-activated protein kinase kinase kinase (MAPKKK) family that activates the JNK and p38 MAPK signaling cascades [110]. ASK1 signaling cascades regulate various cellular responses such as proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis [111].

Accumulating evidence indicates that ASK1 signaling cascade plays an important role in the pathogenesis of Alzheimer's disease. For example, it was reported that ASK1 protein levels were upregulated in both brains of APP transgenic mice [112]. It was also found that Aβ1 increased ASK1 and induce cell death in cultured cells and endothelial cells [113][114].

Many studies also indicated that ASK1 downstream effectors JNK/p38 are activated in brain of AD patients, in AD animal models and by Aβ treatment. It was found that phosphorylated JNK and p38 levels were increased in post-mortem brain tissue of AD patients, and phosphorylated JNK was also co-localized with Aβ deposit [115][116][117]. JNK was also found to be highly expressed and activated in cerebrospinal fluid of AD patients. Activated JNK was correlated with the rate of

cognitive decline [118]. It was found that both JNK and p38 were significantly activated in the cerebral cortex of 7 and 12-month-old *App/Ps1* mice. JNK and p38 activation was also associated with increased levels of Aβ plaques and neurofibrillary tangles in *App/Ps1* mice [119]. Treatment with Aβ was also found to induce JNK activation in primary cultured cortical and hippocampal neurons, as well as SH-SY5Y neuroblastoma cells [120][121][122]. Aβ was also found to activate p38 in primary cultured microglia [123][124].

Accordingly, these studies suggest that ASK1/JNK/p38 signaling cascade is activated and contributes to the pathology of AD. Since Txnip inhibits Trx that is an inhibitor for ASK1, Aβ-increased Txnip may contribute to activation of ASK1 signaling cascade in AD.

### 4.4 Aβ activates NLRP3 signaling

Aβ may upregulate Txnip, increasing Txnip-NLRP3 binding and activating NLRP3 inflammatory signaling. Indeed, many studies have indicated that NLRP3 and its downstream effectors caspase-1 and IL-1β are upregulated in AD patients, AD animal models and Aβ-treated cells.

NLRP3, caspase-1 and IL-1β levels were found to be upregulated in Aβ-treated monocytes from severe and mild AD patients [125]. Caspase-1 levels were also found to

be increased in postmortem hippocampus and frontal cortex of AD patients and 16-month-old *App/Ps1* transgenic mice [126]. It was also noted that IL-1β levels were increased in postmortem temporal cortex of AD patients [127] and that IL-1β levels were also increased in the cerebrospinal fluid of patients with AD [128].

Reactive IL-1β-immunoreactive astrocytes were found in close proximity to both fibrillary and diffuse Aβ deposits in neocortex and hippocampus of 13, 16 and 19 months APP transgenic mice [129]. Aβ has also been found to promote the processing of pro-IL-1β into mature IL-1β, and to induce and promote the processing of pro-IL-1β into mature IL-1β [97][130]. In addition, NLRP3- or caspase-1 deficient *App/Ps1* mice were protected from spatial memory impairment. In the same study, it has also been found that NLRP3- and caspase-1 deficient *App/Ps1* mice showed reduced amounts of Aβ compared with *App/Ps1* mice [126].

These studies indicate that NLRP3 inflammasome and proinflammatory processes are activated and leads to the pathology of AD. Aβ-increased Txnip may accelerate NLRP3 activation. However, a clear role of Txnip in Aβ-induced inflammation needs to be further investigated.

## 4.5 Conclusion

I found that Txnip protein levels were increased in frontal cortex and hippocampus

of 9 and 12-month-old *App/Ps1* transgenic mice. Further, we found that Txnip protein levels were also increased by Aβ in cultured neuronal cells. Txnip inhibits Trx activity, promoting protein cysteine oxidative modifications such as nitrosylation and sulfenylation [70][71]. Previously our laboratory showed that nitrosylated protein levels were increased in frontal cortex and hippocampus of 9 and 12-month-old *App/Ps1* mice [41]. In the present study, we found that sulfenylated protein levels were also increased in frontal cortex and hippocampus of 9 and 12-month-old *App/Ps1* mice. Further treatment with Aβ directly increased protein cysteine nitrosylation and sulfenylation in cultured neuronal cells, and knocking out Txnip inhibited Aβ-induced nitrosylation and sulfenylation.

Because Txnip is an endogenous inhibitor of Trx, our findings suggest that Aβ may increase Txnip, subsequently inhibiting Trx, reducing capability and promoting protein cysteine nitrosylation and sulfenylation, which may contribute to protein oxidative stress and neuronal damage in AD (Figure 23). Because Txnip can bind to Trx and dissociate ASK1 from Trx, resulting in ASK1 activation [73][74]; and because Txnip can also bind to NLRP3, resulting in NLRP3 inflammasome forming [130][149], our study also suggests that Aβ-increased Txnip may facilitate neuronal apoptosis and neuroinflammation in AD. However, whether Aβ-increased Txnip further activates these two pathways requires further investigation.

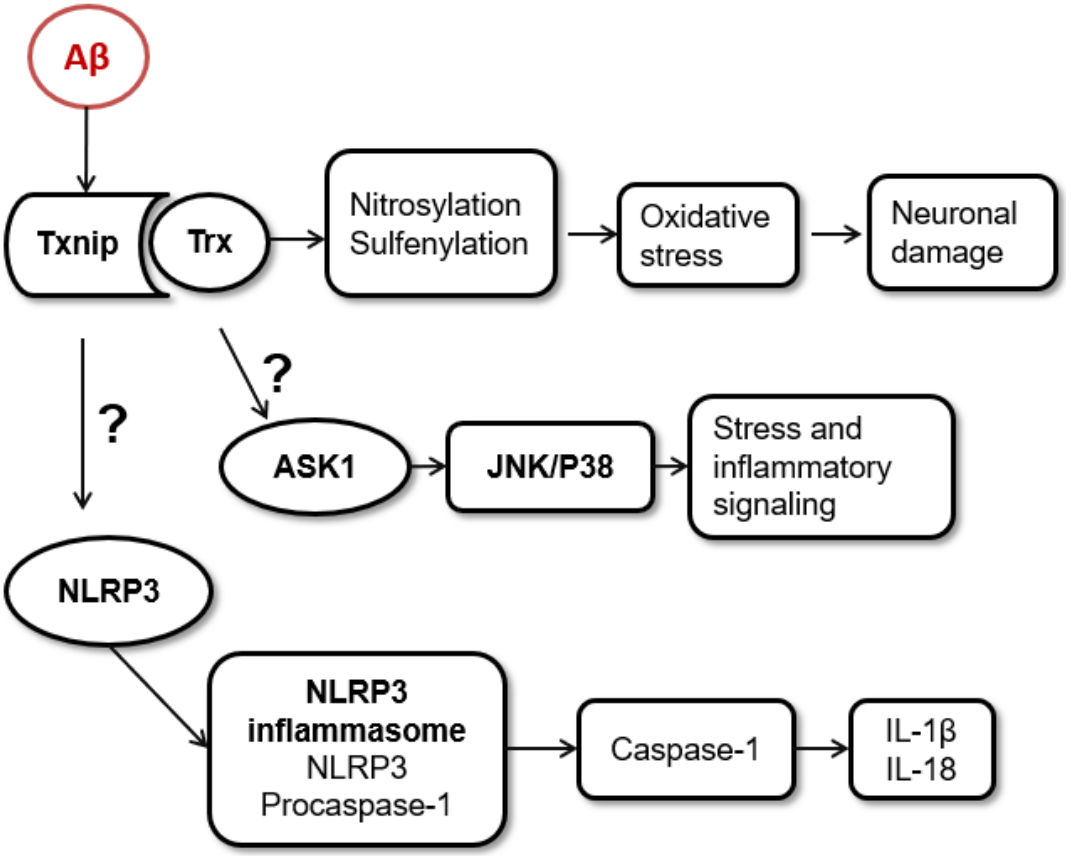


**Figure 23. Summary.** Txnip, Trx-interacting protein; Trx, thioredoxin; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun N-terminal kinase; P38, P38 mitrogen-activated kinase; NLRP3, nod-like receptor protein 3; IL, interleukin

### 4.6 Limitations of the study

In the present study, there are several limitations. First, synthetic human Aβ1-42 peptide was prepared to form Aβ oligomers according to published methods [91]. However, Aβ aggregates often exist as a dynamic mixture of species with a broad range of sizes [173]. They are unstable and may disassemble or assemble during preparation

[174][175]. Because we did not verify the accurate concentration of Aβ oligomers in the stock solution, it is possible that the Aβ solution is a mixture of monomers, oligomers, and protofibrils. I tried to make Aβ preparation consistent in each experiment and repeat at least twice for the *in vitro* study.

Secondly, sulfenylation and nitrosylation are reversible, redox-sensitive and spawn specific posttranslational modifications [176][177]. Therefore, endogenous nitrosylation and sulfenylation detection are technically more challenging than some other modifications such as phosphorylation and carbonylation. Also, the nitrosylated thiol group and sulfenic acid are both light sensitive, which makes analysis more difficult [41].

Thirdly, various brain regions contribute to AD development. However, I only measured the effect of Aβ in Txnip protein levels in primary cultured mouse cerebral cortical neurons, but not other regions. In the future, Txnip regulation in other brain regions will be explored.

## BIBLIOGRAPHY


[1] Alzheimer's Association. (2017). 2017 Alzheimer's disease facts and figures. *Alzheimer's & Dementia*, *13*(4), 325-373.

[2] Glenner, G. G., & Wong, C. W. (1984). Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochemical and biophysical research communications*, *120*(3), 885-890.

[3] Masters, C. L., Simms, G., Weinman, N. A., Multhaup, G., McDonald, B. L., & Beyreuther, K. (1985). Amyloid plaque core protein in Alzheimer disease and Down syndrome. *Proceedings of the National Academy of Sciences*, *82*(12), 4245-4249.

[4] Gorevic, P. D., Goñi, F., Pons-Estel, B., Alvarez, F., Peress, N. S., & Frangione, B. (1986). Isolation and partial characterization of neurofibrillary tangles and amyloid plaque core in Alzheimer's disease: immunohistological studies. *Journal of Neuropathology & Experimental Neurology*, *45*(6), 647-664.

[5] Hardy, J., & Allsop, D. (1991). Amyloid deposition as the central event in the aetiology of Alzheimer's disease. *Trends in pharmacological sciences*, *12*, 383-388.

[6] Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., ... & Selkoe, D. J. (1992). Mutation of the β-amyloid precursor protein in familial Alzheimer's disease increases β-protein production. *Nature*, *360*(6405), 672.

[7] Cai, X. D., Golde, T. E., & Younkin, S. G. (1993). Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. *Science*, *259*(5094), 514-516.

[8] Sherrington, R., Rogaev, E. I., Liang, Y. A., Rogaeva, E. A., Levesque, G., Ikeda, M., ... & Tsuda, T. (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. *Nature*, *375*(6534), 754.

[9] Selkoe, D. J. (1991). The molecular pathology of Alzheimer's disease. *Neuron, 6*(4), 487-498.

[10] Hardy, J. A., & Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science*, *256*(5054), 184.

[11] Tucker, R. P. (1990). The roles of microtubule-associated proteins in brain morphogenesis: a review. *Brain Research Reviews*, *15*(2), 101-120.

[12] Lee, G., Neve, R. L., & Kosik, K. S. (1989). The microtubule binding domain of tau protein. *Neuron*, *2*(6), 1615-1624.

[13] Kuret, J., Chirita, C. N., Congdon, E. E., Kannanayakal, T., Li, G., Necula, M., ... & Zhong, Q. (2005). Pathways of tau fibrillization. *Biochimica Et Biophysica Acta (BBA)-Molecular Basis of Disease*, *1739*(2-3), 167-178.

[14] Köpke, E., Tung, Y. C., Shaikh, S., Alonso, A. D. C., Iqbal, K., & Grundke-Iqbal, I. (1993). Microtubule-associated protein tau. Abnormal phosphorylation of a non-paired helical filament pool in Alzheimer disease. *Journal of Biological*

*Chemistry*, *268*(32), 24374-24384.

[15] Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., & Binder, L. I. (1986). Abnormal phosphorylation of the microtubule-associated protein tau (tau) in Alzheimer cytoskeletal pathology. *Proceedings of the National Academy of Sciences*, *83*(13), 4913-4917.

[16] Roy, S., Zhang, B., Lee, V. M. Y., & Trojanowski, J. Q. (2005). Axonal transport defects: a common theme in neurodegenerative diseases. *Acta neuropathologica*, *109*(1), 5-13.

[17] Bowen, D. M., Smith, C. B., White, P. A. M. E. L. A., & Davison, A. N. (1976). Neurotransmitter-related enzymes and indices of hypoxia in senile dementia and other abiotrophies. *Brain: a journal of neurology*, *99*(3), 459-496.

[18] Davies, P., & Maloney, A. J. F. (1976). Selective loss of central cholinergic neurons in Alzheimer's disease. *The Lancet*, *308*(8000), 1403.

[19] Kar, S., Issa, A. M., Seto, D., Auld, D. S., Collier, B., & Quirion, R. (1998). Amyloid β Peptide Inhibits High Affinity Choline Uptake and Acetylcholine Release in Rat Hippocampal Slices. *Journal of neurochemistry*, *70*(5), 2179-2187.

[20] Auld, D. S., Kar, S., & Quirion, R. (1998). β-Amyloid peptides as direct cholinergic neuromodulators: a missing link? *Trends in neurosciences*, *21*(1), 43-49.

[21] Auld, D. S., Kornecook, T. J., Bastianetto, S., & Quirion, R. (2002). Alzheimer's disease and the basal forebrain cholinergic system: relations to β-amyloid peptides, cognition, and treatment strategies. *Progress in neurobiology*, *68*(3), 209-245.

[22] Liu, Y., Zhang, Y. W., Wang, X., Zhang, H., You, X., Liao, F. F., & Xu, H. (2009). Intracellular trafficking of presenilin 1 is regulated by β-amyloid precursor protein and phospholipase D1. *Journal of Biological Chemistry*, *284*(18), 12145-12152.

[23] Allsop, D., Landon, M., & Kidd, M. (1983). The isolation and amino acid composition of senile plaque core protein. *Brain research*, *259*(2), 348-352.

[24] Bayer, T. A., Wirths, O., Majtényi, K., Hartmann, T., Multhaup, G., Beyreuther, K., & Czech, C. (2001). Key factors in Alzheimer's disease: β-amyloid precursor protein processing, metabolism and intraneuronal transport. *Brain Pathology*, *11*(1), 1-11.

[25] Vetrivel, K. S., & Thinakaran, G. (2006). Amyloidogenic processing of β-amyloid precursor protein in intracellular compartments. *Neurology*, *66*(1 suppl 1), S69-S73.

[26] Buchhave, P., Minthon, L., Zetterberg, H., Wallin, Å. K., Blennow, K., & Hansson, O. (2012). Cerebrospinal fluid levels ofβ-amyloid 1-42, but not of tau, are fully changed already 5 to 10 years before the onset of Alzheimer dementia. *Archives of general psychiatry*, *69*(1), 98-106.

[27] Bateman, R. J., Xiong, C., Benzinger, T. L., Fagan, A. M., Goate, A., Fox, N. C., ... & Holtzman, D. M. (2012). Clinical and biomarker changes in dominantly inherited Alzheimer's disease. *New England Journal of Medicine*, *367*(9), 795-804.

[28] Cerasoli, E., Ryadnov, M., & Austen, B. M. (2015). The elusive nature and diagnostics of misfolded Aβ oligomers. *Frontiers in chemistry*, *3*, 17.

[29] Paulson, J. B., Ramsden, M., Forster, C., Sherman, M. A., McGowan, E., & Ashe, K. H. (2008). Amyloid plaque and neurofibrillary tangle pathology in a regulatable mouse model of Alzheimer's disease. *The American journal of pathology*, *173*(3), 762-772.

[30] Goate, A., Chartier-Harlin, M. C., Mullan, M., Brown, J., Crawford, F., Fidani, L., ... & Mant, R. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature*, *349*(6311), 704.

[31] Suzuki, N., Cheung, T. T., Cai, X. D., Odaka, A., Otvos, L., Eckman, C., ... & Younkin, S. G. (1994). An increased percentage of long amyloid beta protein secreted by familial amyloid beta protein precursor (beta APP717) mutants. *Science*, *264*(5163), 1336-1340.

[32] Rogaev, E. I., Sherrington, R., Rogaeva, E. A., Levesque, G., Ikeda, M., Liang, Y., ... & Mar, L. (1995). Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. *Nature*, *376*(6543), 775.

[33] De Strooper, B., Saftig, P., Craessaerts, K., Vanderstichele, H., Guhde, G., Annaert, W., ... & Van Leuven, F. (1998). Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature*, *391*(6665), 387.

[34] Bertram, L., & Tanzi, R. E. (2004). The current status of Alzheimer's disease genetics: what do we tell the patients? *Pharmacological research*, *50*(4), 385-396.

[35] Duff, K. (1997). Alzheimer transgenic mouse models come of age. *Trends in neurosciences*, *20*(7), 279-280.

[36] Oyama, F., Sawamura, N., Kobayashi, K., Morishima-Kawashima, M., Kuramochi, T., Ito, M., ... & Capell, A. (1998). Mutant presenilin 2 transgenic mouse: Effect on an age dependent increase of amyloid β protein 42 in the brain. *Journal of neurochemistry*, *71*(1), 313-322.

[37] Sherrington, R., Froelich, S., Sorbi, S., Campion, D., Chi, H., Rogaeva, E. A., ... & Ikeda, M. (1996). Alzheimer's disease associated with mutations in presenilin 2 is rare and variably penetrant. *Human molecular genetics*, *5*(7), 985-988.

[38] Holcomb, L. A., Gordon, M. N., Jantzen, P., Hsiao, K., Duff, K., & Morgan, D. (1999). Behavioral changes in transgenic mice expressing both amyloid precursor protein and presenilin-1 mutations: lack of association with amyloid deposits. *Behavior genetics*, *29*(3), 177-185.

[39] Dineley, K. T., Xia, X., Bui, D., Sweatt, J. D., & Zheng, H. (2002). Accelerated plaque accumulation, associative learning deficits, and up-regulation of α7 nicotinic receptor protein in transgenic mice co-expressing mutant human presenilin 1 and

amyloid precursor proteins. *Journal of Biological Chemistry*, *277*(25), 22768-22780.

[40] Cramer, P. E., Cirrito, J. R., Wesson, D. W., Lee, C. D., Karlo, J. C., Zinn, A. E., ... & Brunden, K. R. (2012). ApoE-directed therapeutics rapidly clear β-amyloid and reverse deficits in AD mouse models. *science*, *335*(6075), 1503-1506.

[41] Wang, Y., Zhou, Z., Tan, H., Zhu, S., Wang, Y., Sun, Y., ... & Wang, J. F. (2017). Nitrosylation of Vesicular Transporters in Brain of Amyloid Precursor Protein/Presenilin 1 Double Transgenic Mice. *Journal of Alzheimer's Disease*, *55*(4), 1683-1692.

[42] Glabe, C. G., & Kayed, R. (2006). Common structure and toxic function of amyloid oligomers implies a common mechanism of pathogenesis. *Neurology*, *66*(1 suppl 1), S74-S78.

[43] Reddy, P. H., & Beal, M. F. (2008). Amyloid beta, mitochondrial dysfunction and synaptic damage: implications for cognitive decline in aging and Alzheimer's disease. *Trends in molecular medicine*, *14*(2), 45-53.

[44] Baldeiras, I., Santana, I., Proença, M. T., Garrucho, M. H., Pascoal, R., Rodrigues, A., ... & Oliveira, C. R. (2008). Peripheral oxidative damage in mild cognitive impairment and mild Alzheimer's disease. *Journal of Alzheimer's Disease*, *15*(1), 117-128.

[45] Greilberger, J., Koidl, C., Greilberger, M., Lamprecht, M., Schroecksnadel, K., Leblhuber, F., ... & Oettl, K. (2008). Malondialdehyde, carbonyl proteins and albumin-disulphide as useful oxidative markers in mild cognitive impairment and Alzheimer's disease. *Free radical research*, *42*(7), 633-638.

[46] Bradley, M. A., Xiong-Fister, S., Markesbery, W. R., & Lovell, M. A. (2012). Elevated 4-hydroxyhexenal in Alzheimer's disease (AD) progression. *Neurobiology of aging*, *33*(6), 1034-1044.

[47] Markesbery, W. R., Kryscio, R. J., Lovell, M. A., & Morrow, J. D. (2005). Lipid peroxidation is an early event in the brain in amnestic mild cognitive impairment. *Annals of neurology*, *58*(5), 730-735.

[48] Ansari, M. A., & Scheff, S. W. (2010). Oxidative stress in the progression of Alzheimer disease in the frontal cortex. *Journal of Neuropathology & Experimental Neurology*, *69*(2), 155-167.

[49] Lovell, M. A., Gabbita, S. P., & Markesbery, W. R. (1999). Increased DNA oxidation and decreased levels of repair products in Alzheimer's disease ventricular CSF. *Journal of neurochemistry*, *72*(2), 771-776.

[50] Lovell, M. A., Xie, C., & Markesbery, W. R. (2001). Acrolein is increased in Alzheimer’s disease brain and is toxic to primary hippocampal cultures. *Neurobiology of aging*, *22*(2), 187-194.

[51] Resende, R., Moreira, P. I., Proença, T., Deshpande, A., Busciglio, J., Pereira, C., &

Oliveira, C. R. (2008). Brain oxidative stress in a triple-transgenic mouse model of Alzheimer disease. *Free Radical Biology and Medicine*, *44*(12), 2051-2057.

[52] Praticò, D., Uryu, K., Leight, S., Trojanoswki, J. Q., & Lee, V. M. Y. (2001). Increased lipid peroxidation precedes amyloid plaque formation in an animal model of Alzheimer amyloidosis. *Journal of Neuroscience*, *21*(12), 4183-4187.

[53] Feng, Z., Qin, C., Chang, Y., & Zhang, J. T. (2006). Early melatonin supplementation alleviates oxidative stress in a transgenic mouse model of Alzheimer's disease. *Free Radical Biology and Medicine*, *40*(1), 101-109.

[54] Apelt, J., Bigl, M., Wunderlich, P., & Schliebs, R. (2004). Aging-related increase in oxidative stress correlates with developmental pattern of beta-secretase activity and beta-amyloid plaque formation in transgenic Tg2576 mice with Alzheimer-like pathology. *International Journal of Developmental Neuroscience*, *22*(7), 475-484.

[55] Lim, G. P., Chu, T., Yang, F., Beech, W., Frautschy, S. A., & Cole, G. M. (2001). The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *Journal of Neuroscience*, *21*(21), 8370-8377.

[56] Canevari, L., Clark, J. B., & Bates, T. E. (1999). β-Amyloid fragment 25–35 selectively decreases complex IV activity in isolated mitochondria. *FEBS letters*, *457*(1), 131-134.

[57] Casley, C. S., Land, J. M., Sharpe, M. A., Clark, J. B., Duchen, M. R., & Canevari, L. (2002). β-Amyloid fragment 25–35 causes mitochondrial dysfunction in primary cortical neurons. *Neurobiology of disease*, *10*(3), 258-267.

[58] Behl, C., Davis, J. B., Lesley, R., & Schubert, D. (1994). Hydrogen peroxide mediates amyloid β protein toxicity. *Cell*, *77*(6), 817-827.

[59] De Felice, F. G., Velasco, P. T., Lambert, M. P., Viola, K., Fernandez, S. J., Ferreira, S. T., & Klein, W. L. (2007). Aβ oligomers induce neuronal oxidative stress through an N-methyl-D-aspartate receptor-dependent mechanism that is blocked by the Alzheimer drug memantine. *Journal of Biological Chemistry*, *282*(15), 11590-11601.

[60] Giles, N. M., Watts, A. B., Giles, G. I., Fry, F. H., Littlechild, J. A., & Jacob, C. (2003). Metal and redox modulation of cysteine protein function. *Chemistry & biology*, *10*(8), 677-693.

[61] He, X., & Ma, Q. (2009). Induction of Metallothionein I by arsenic via metal-activated transcription factor 1 critical role of C-terminal cysteine residues in arsenic sensing. *Journal of Biological Chemistry*, *284*(19), 12609-12621.

[62] Nakamura, T., Tu, S., Akhtar, M. W., Sunico, C. R., Okamoto, S. I., & Lipton, S. A. (2013). Aberrant protein s-nitrosylation in neurodegenerative diseases. *Neuron*, *78*(4), 596-614.

[63] Wang, Y., Zhou, Z., Leylek, T., Tan, H., Sun, Y., Parkinson, F. E., & Wang, J. F. (2015). Protein cysteine S-nitrosylation inhibits vesicular uptake of

neurotransmitters. *Neuroscience*, *311*, 374-381.

[64] Malito, E., Hulse, R. E., & Tang, W. J. (2008). Amyloid β-degrading cryptidases: insulin degrading enzyme, presequence peptidase, and neprilysin. *Cellular and Molecular Life Sciences*, *65*(16), 2574-2585.

[65] Jiang, Q., Lee, C. D., Mandrekar, S., Wilkinson, B., Cramer, P., Zelcer, N., ... & Richardson, J. C. (2008). ApoE promotes the proteolytic degradation of Aβ. *Neuron*, *58*(5), 681-693.

[66] Kwak, Y. D., Wang, R., Li, J. J., Zhang, Y. W., Xu, H., & Liao, F. F. (2011). Differential regulation of BACE1 expression by oxidative and nitrosative signals. *Molecular neurodegeneration*, *6*(1), 17.

[67] Kruszelnicka, O. (2014). Nitric oxide vs insulin secretion, action and clearance. *Diabetologia*, *57*(1), 257-258.

[68] Abrams, A. J., Farooq, A., & Wang, G. (2011). S-nitrosylation of ApoE in Alzheimer's disease. *Biochemistry*, *50*(17), 3405-3407.

[69] Cordes, C. M., Bennett, R. G., Siford, G. L., & Hamel, F. G. (2009). Nitric oxide inhibits insulin-degrading enzyme activity and function through S-nitrosylation. *Biochemical pharmacology*, *77*(6), 1064-1073.

[70] Laurent, T. C., Moore, E. C., & Reichard, P. (1964). Enzymatic synthesis of deoxyribonucleotides IV. Isolation and characterization of thioredoxin, the hydrogen donor from Escherichia coli B. *Journal of Biological Chemistry*, *239*(10), 3436-3444.

[71] Wu, C., Parrott, A. M., Liu, T., Jain, M. R., Yang, Y., Sadoshima, J., & Li, H. (2011). Distinction of thioredoxin transnitrosylation and denitrosylation target proteins by the ICAT quantitative approach. *Journal of proteomics*, *74*(11), 2498-2509.

[72] Kang, S. W., Baines, I. C., & Rhee, S. G. (1998). Characterization of a mammalian peroxiredoxin that contains one conserved cysteine. *Journal of Biological Chemistry*, *273*(11), 6303-6311.

[73] Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., ... & Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *The EMBO journal*, *17*(9), 2596-2606.

[74] Liu, Y., & Min, W. (2002). Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circulation research*, *90*(12), 1259-1266.

[75] Matthews, J. R., Wakasugi, N., Virelizier, J. L., Yodoi, J., & Hay, R. T. (1992). Thiordoxin regulates the DNA binding activity of NF-χB by reduction of a disulphid bond involving cysteine 62. *Nucleic acids research*, *20*(15), 3821-3830.

[76] Hayashi, T., Ueno, Y., & Okamoto, T. (1993). Oxidoreductive regulation of nuclear factor kappa B. Involvement of a cellular reducing catalyst thioredoxin. *Journal of*

*Biological Chemistry*, *268*(15), 11380-11388.

[77] Schenk, H., Klein, M., Erdbrügger, W., Dröge, W., & Schulze-Osthoff, K. (1994). Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proceedings of the National Academy of Sciences*, *91*(5), 1672-1676.

[78] Zhong, L., Arnér, E. S., & Holmgren, A. (2000). Structure and mechanism of mammalian thioredoxin reductase: the active site is a redox-active selenolthiol/selenenylsulfide formed from the conserved cysteine-selenocysteine sequence. *Proceedings of the National Academy of Sciences*, *97*(11), 5854-5859.

[79] Zhong, L., Arnér, E. S., Ljung, J., Åslund, F., & Holmgren, A. (1998). Rat and calf thioredoxin reductase are homologous to glutathione reductase with a carboxyl-terminal elongation containing a conserved catalytically active penultimate selenocysteine residue. *Journal of Biological Chemistry*, *273*(15), 8581-8591.

[80] Haendeler, J. (2006). Thioredoxin-1 and posttranslational modifications. *Antioxidants & redox signaling*, *8*(9-10), 1723-1728.

[81] Gaston, B. M., Carver, J., Doctor, A., & Palmer, L. A. (2003). S-nitrosylation signaling in cell biology. *Molecular interventions*, *3*(5), 253.

[82] Haendeler, J., Hoffmann, J., Tischler, V., Berk, B. C., Zeiher, A. M., & Dimmeler, S. (2002). Redox regulatory and anti-apoptotic functions of thioredoxin depend on S-nitrosylation at cysteine 69. *Nature cell biology*, *4*(10), 743.

[83] Moi, P., Chan, K., Asunis, I., Cao, A., & Kan, Y. W. (1994). Isolation of NF-E2-related factor 2 (Nrf2), a NF-E2-like basic leucine zipper transcriptional activator that binds to the tandem NF-E2/AP1 repeat of the beta-globin locus control region. *Proceedings of the National Academy of Sciences*, *91*(21), 9926-9930.

[84] Nguyen, T., Sherratt, P. J., & Pickett, C. B. (2003). Regulatory mechanisms controlling gene expression mediated by the antioxidant response element. *Annual review of pharmacology and toxicology*, *43*(1), 233-260.

[85] Copple, I. M. (2012). The Keap1–Nrf2 cell defense pathway–a promising therapeutic target? In *Advances in pharmacology* (Vol. 63, pp. 43-79). Academic Press.

[86] Choi, J., Sullards, M. C., Olzmann, J. A., Rees, H. D., Weintraub, S. T., Bostwick, D. E., ... & Li, L. (2006). Oxidative damage of DJ-1 is linked to sporadic Parkinson and Alzheimer diseases. *Journal of Biological Chemistry*, *281*(16), 10816-10824.

[87] Bandopadhyay, R., Kingsbury, A. E., Cookson, M. R., Reid, A. R., Evans, I. M., Hope, A. D., ... & McLendon, C. (2004). The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. *Brain*, *127*(2), 420-430.

[88] Im, J. Y., Lee, K. W., Woo, J. M., Junn, E., & Mouradian, M. M. (2012). DJ-1 induces thioredoxin 1 expression through the Nrf2 pathway. *Human molecular genetics*, *21*(13), 3013-3024.

[89] Akterin, S., Cowburn, R. F., Miranda-Vizuete, A., Jiménez, A., Bogdanovic, N., Winblad, B., & Cedazo-Minguez, A. (2006). Involvement of glutaredoxin-1 and thioredoxin-1 in β-amyloid toxicity and Alzheimer's disease. *Cell death and differentiation*, *13*(9), 1454.

[90] Lovell, M. A., Xie, C., Gabbita, S. P., & Markesbery, W. R. (2000). Decreased thioredoxin and increased thioredoxin reductase levels in Alzheimer's disease brain. *Free Radical Biology and Medicine*, *28*(3), 418-427.

[91] Batarseh, Y. S., Mohamed, L. A., Al Rihani, S. B., Mousa, Y. M., Siddique, A. B., El Sayed, K. A., & Kaddoumi, A. (2017). Oleocanthal ameliorates amyloid-β oligomers' toxicity on astrocytes and neuronal cells: In vitro studies. *Neuroscience*, *352*, 204-215.

[92] Wang, H., Yu, S. W., Koh, D. W., Lew, J., Coombs, C., Bowers, W., ... & Dawson, V. L. (2004). Apoptosis-Inducing Factor Substitutes for Caspase Executioners in NMDA-Triggered Excitotoxic Neuronal Death. *The Journal of Neuroscience*, *24*(48), 10963-10973.

[93] Bharti, V., Tan, H., Chow, D., Wang, Y., Nagakannan, P., Eftekharpour, E., & Wang, J. F. (2018). Glucocorticoid upregulates thioredoxin-interacting protein in cultured neuronal cells. *Neuroscience*.

[94] Nagakannan, P., Iqbal, M. A., Yeung, A., Thliveris, J. A., Rastegar, M., Ghavami, S., & Eftekharpour, E. (2016). Perturbation of redox balance after thioredoxin reductase deficiency interrupts autophagy-lysosomal degradation pathway and enhances cell death in nutritionally stressed SH-SY5Y cells. *Free Radical Biology and Medicine*, *101*, 53-70.

[95] Gouget, T., Djelloul, M., Boucraut, J., Weinhard, L., Baranger, K., Rivera, S., ... & Perrone, L. (2011). TXNIP, the major player in insulin resistance, is early over-expressed in the brain of the 5XFAD Alzheimer's mice model and is induced by Aβ in vitro: emerging role of TXNIP and inflammation in Alzheimer's Disease progression. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*, *7*(4), S684.

[96] Oakley, H., Cole, S. L., Logan, S., Maus, E., Shao, P., Craft, J., ... & Berry, R. (2006). Intraneuronal β-amyloid aggregates, neurodegeneration, and neuron loss in transgenic mice with five familial Alzheimer's disease mutations: potential factors in amyloid plaque formation. *Journal of Neuroscience*, *26*(40), 10129-10140.

[97] Parajuli, B., Sonobe, Y., Horiuchi, H., Takeuchi, H., Mizuno, T., & Suzumura, A. (2013). Oligomeric amyloid β induces IL-1β processing via production of ROS: implication in Alzheimer's disease. *Cell death & disease*, *4*(12), e975.

[98] Piccini, A., Borghi, R., Guglielmotto, M., Tamagno, E., Cirmena, G., Garuti, A., ... & Colombo, L. (2012). β-Amyloid 1-42 induces physiological transcriptional

regulation of BACE1. *Journal of neurochemistry*, *122*(5), 1023-1031.

[99] Gil-Bea, F., Akterin, S., Persson, T., Mateos, L., Sandebring, A., Avila-Cariño, J., ... & Cedazo-Minguez, A. (2012). Thioredoxin-80 is a product of alpha-secretase cleavage that inhibits amyloid-beta aggregation and is decreased in Alzheimer's disease brain. *EMBO molecular medicine*, *4*(10), 1097-1111.

[100] Spindel, O. N., World, C., & Berk, B. C. (2012). Thioredoxin interacting protein: redox dependent and independent regulatory mechanisms. *Antioxidants & redox signaling*, *16*(6), 587-596.

[101] Chong, C. R., Chan, W. P. A., Nguyen, T. H., Liu, S., Procter, N. E., Ngo, D. T., ... & Horowitz, J. D. (2014). Thioredoxin-interacting protein: pathophysiology and emerging pharmacotherapeutics in cardiovascular disease and diabetes. *Cardiovascular drugs and therapy*, *28*(4), 347-360.

[102] Zhou, R., Yazdi, A. S., Menu, P., & Tschopp, J. (2011). A role for mitochondria in NLRP3 inflammasome activation. *Nature*, *469*(7329), 221.

[103] Oslowski, C. M., Hara, T., O'Sullivan-Murphy, B., Kanekura, K., Lu, S., Hara, M., ... & Greiner, D. (2012). Thioredoxin-interacting protein mediates ER stress-induced β cell death through initiation of the inflammasome. *Cell metabolism*, *16*(2), 265-273.

[104] Lee, T. H., Pastorino, L., & Lu, K. P. (2011). Peptidyl-prolyl cis–trans isomerase Pin1 in ageing, cancer and Alzheimer disease. *Expert reviews in molecular medicine*, *13*.

[105] Liou, Y. C., Zhou, X. Z., & Lu, K. P. (2011). Prolyl isomerase Pin1 as a molecular switch to determine the fate of phosphoproteins. *Trends in biochemical sciences*, *36*(10), 501-514.

[106] Sultana, R., Poon, H. F., Cai, J., Pierce, W. M., Merchant, M., Klein, J. B., ... & Butterfield, D. A. (2006). Identification of nitrated proteins in Alzheimer's disease brain using a redox proteomics approach. *Neurobiology of disease*, *22*(1), 76-87.

[107] Dong, Y. T., Cao, K., Tan, L. C., Wang, X. L., Qi, X. L., Xiao, Y., & Guan, Z. Z. (2018). Stimulation of SIRT1 Attenuates the Level of Oxidative Stress in the Brains of *App/Ps1* Double Transgenic Mice and in Primary Neurons Exposed to Oligomers of the Amyloid-β Peptide. *Journal of Alzheimer's Disease*, (Preprint), 1-19.

[108] Hashimoto, M., Tozawa, R., Katakura, M., Shahdat, H., Haque, A. M., Tanabe, Y., ... & Shido, O. (2011). Protective effects of prescription n-3 fatty acids against impairment of spatial cognitive learning ability in amyloid β-infused rats. *Food & function*, *2*(7), 386-394.

[109] D Rege, S., Geetha, T., L Broderick, T., & Ramesh Babu, J. (2015). Resveratrol protects β amyloid-induced oxidative damage and memory associated proteins in H19-7 hippocampal neuronal cells. *Current Alzheimer Research*, *12*(2), 147-156.

[110] Kawarazaki, Y., Ichijo, H., & Naguro, I. (2014). Apoptosis signal-regulating kinase 1

as a therapeutic target. *Expert opinion on therapeutic targets*, *18*(6), 651-664.

[111] Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., & Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. *Endocrine reviews*, *22*(2), 153-183.

[112] Galvan, V., Banwait, S., Spilman, P., Gorostiza, O. F., Peel, A., Ataie, M., ... & Bredesen, D. E. (2007). Interaction of ASK1 and the β-amyloid precursor protein in a stress-signaling complex. *Neurobiology of disease*, *28*(1), 65-75.

[113] Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., ... & Ichijo, H. (2005). Amyloid β induces neuronal cell death through ROS-mediated ASK1 activation. *Cell death and differentiation*, *12*(1), 19.

[114] Hsu, M. J., Hsu, C. Y., Chen, B. C., Chen, M. C., Ou, G., & Lin, C. H. (2007). Apoptosis signal-regulating kinase 1 in amyloid β peptide-induced cerebral endothelial cell apoptosis. *Journal of Neuroscience*, *27*(21), 5719-5729.

[115] Zhu, X., Castellani, R. J., Takeda, A., Nunomura, A., Atwood, C. S., Perry, G., & Smith, M. A. (2001). Differential activation of neuronal ERK, JNK/SAPK and p38 in Alzheimer disease: the 'two hit'hypothesis. *Mechanisms of ageing and development*, *123*(1), 39-46.

[116] Killick, R., Ribe, E. M., Al-Shawi, R., Malik, B., Hooper, C., Fernandes, C., ... & Lin, K. (2014). Clusterin regulates β-amyloid toxicity via Dickkopf-1-driven induction of the wnt–PCP–JNK pathway. *Molecular psychiatry*, *19*(1), 88.

[117] Sun, H. Y., Wang, N. P., Halkos, M., Kerendi, F., Kin, H., Guyton, R. A., ... & Zhao, Z. Q. (2006). Postconditioning attenuates cardiomyocyte apoptosis via inhibition of JNK and p38 mitogen-activated protein kinase signaling pathways. *Apoptosis*, *11*(9), 1583-1593.

[118] Gourmaud, S., Paquet, C., Dumurgier, J., Pace, C., Bouras, C., Gray, F., ... & Hugon, J. (2015). Increased levels of cerebrospinal fluid JNK3 associated with amyloid pathology: links to cognitive decline. *Journal of psychiatry & neuroscience: JPN*, *40*(3), 151.

[119] Savage, M. J., Lin, Y. G., Ciallella, J. R., Flood, D. G., & Scott, R. W. (2002). Activation of c-Jun N-terminal kinase and p38 in an Alzheimer's disease model is associated with amyloid deposition. *Journal of Neuroscience*, *22*(9), 3376-3385.

[120] Morishima, Y., Gotoh, Y., Zieg, J., Barrett, T., Takano, H., Flavell, R., ... & Greenberg, M. E. (2001). β-Amyloid induces neuronal apoptosis via a mechanism that involves the c-Jun N-terminal kinase pathway and the induction of Fas ligand. *Journal of Neuroscience*, *21*(19), 7551-7560.

[121] Suwanna, N., Thangnipon, W., & Soi-ampornkul, R. (2014). Neuroprotective effects of diarylpropionitrile against β-amyloid peptide-induced neurotoxicity in rat cultured cortical neurons. *Neuroscience letters*, *578*, 44-49.

[122] Xu, K., Chen, W., Wang, X., Peng, Y., Liang, A., Huang, D., ... & Ye, W. (2015). Autophagy attenuates the catabolic effect during inflammatory conditions in nucleus pulposus cells, as sustained by NF-κB and JNK inhibition. *International journal of molecular medicine*, *36*(3), 661-668.

[123] McDonald, D. R., Bamberger, M. E., Combs, C. K., & Landreth, G. E. (1998). β-Amyloid fibrils activate parallel mitogen-activated protein kinase pathways in microglia and THP1 monocytes. *Journal of Neuroscience*, *18*(12), 4451-4460.

[124] Kim, S. O., Chun, K. S., Kundu, J. K., & Surh, Y. J. (2004). Inhibitory effects of [6]-gingerol on PMA-induced COX-2 expression and activation of NF-κB and p38 MAPK in mouse skin. *Biofactors*, *21*(1-4), 27-31.

[125] Saresella, M., La Rosa, F., Piancone, F., Zoppis, M., Marventano, I., Calabrese, E., ... & Clerici, M. (2016). The NLRP3 and NLRP1 inflammasomes are activated in Alzheimer's disease. *Molecular neurodegeneration*, *11*(1), 23.

[126] Heneka, M. T., Kummer, M. P., Stutz, A., Delekate, A., Schwartz, S., Vieira-Saecker, A., ... & Gelpi, E. (2013). NLRP3 is activated in Alzheimer's disease and contributes to pathology in *App/Ps1* mice. *Nature*, *493*(7434), 674.

[127] Griffin, W. S., Stanley, L. C., Ling, C. H. E. N., White, L., MacLeod, V., Perrot, L. J., ... & Araoz, C. (1989). Brain interleukin 1 and S-100 immunoreactivity are elevated in Down syndrome and Alzheimer disease. *Proceedings of the National Academy of Sciences*, *86*(19), 7611-7615.

[128] Blum-Degena, D., Müller, T., Kuhn, W., Gerlach, M., Przuntek, H., & Riederer, P. (1995). Interleukin-1β and interleukin-6 are elevated in the cerebrospinal fluid of Alzheimer's and de novo Parkinson's disease patients. *Neuroscience letters*, *202*(1-2), 17-20.

[129] Apelt, J., & Schliebs, R. (2001). β-amyloid-induced glial expression of both pro-and anti-inflammatory cytokines in cerebral cortex of aged transgenic Tg2576 mice with Alzheimer plaque pathology. *Brain research*, *894*(1), 21-30.

[130] Halle, A., Hornung, V., Petzold, G. C., Stewart, C. R., Monks, B. G., Reinheckel, T., ... & Golenbock, D. T. (2008). The NALP3 inflammasome is involved in the innate immune response to amyloid-β. *Nature immunology*, *9*(8), 857.

[131] Dodd, P. R., Scott, H. L., & Westphalen, R. I. (1994). Excitotoxic mechanisms in the pathogenesis of dementia. *Neurochemistry international*, *25*(3), 203-219.

[132] Watkins, J. C., & Evans, R. H. (1981). Excitatory amino acid transmitters. *Annual review of pharmacology and toxicology*, *21*(1), 165-204.

[133] Butterfield, D. A., & Pocernich, C. B. (2003). The glutamatergic system and Alzheimer's disease. *CNS drugs*, *17*(9), 641-652.

[134] Wang, Y., & Qin, Z. H. (2010). Molecular and cellular mechanisms of excitotoxic neuronal death. *Apoptosis*, *15*(11), 1382-1402.

[135] Cosgrove, G. R., Leblanc, R., Meagher-Villemure, K., & Ethier, R. (1985). Cerebral amyloid angiopathy. *Neurology*, *35*(5), 625-625.

[136] Cosgrove, G. R., Leblanc, R., Meagher-Villemure, K., & Ethier, R. (1985). Cerebral amyloid angiopathy. *Neurology*, *35*(5), 625-625.

[137] Wu, C., Parrott, A. M., Fu, C., Liu, T., Marino, S. M., Gladyshev, V. N., ... & Sadoshima, J. (2011). Thioredoxin 1-mediated post-translational modifications: reduction, transnitrosylation, denitrosylation, and related proteomics methodologies. *Antioxidants & redox signaling*, *15*(9), 2565-2604.

[138] Matsui, M., Oshima, M., Oshima, H., Takaku, K., Maruyama, T., Yodoi, J., & Taketo, M. M. (1996). Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Developmental biology*, *178*(1), 179-185.

[139] Nonn, L., Williams, R. R., Erickson, R. P., & Powis, G. (2003). The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice. *Molecular and cellular biology*, *23*(3), 916-922.

[140] Soerensen, J., Jakupoglu, C., Beck, H., Förster, H., Schmidt, J., Schmahl, W., ... & Brielmeier, M. (2008). The role of thioredoxin reductases in brain development. *PLoS One*, *3*(3), e1813.

[141] Chutkow, W. A., Patwari, P., Yoshioka, J., & Lee, R. T. (2007). Txnip is a critical regulator of hepatic glucose production. *Journal of Biological Chemistry*.

[142] Hui, S. T., Andres, A. M., Miller, A. K., Spann, N. J., Potter, D. W., Post, N. M., ... & Davis, R. A. (2008). Txnip balances metabolic and growth signaling via PTEN disulfide reduction. *Proceedings of the National Academy of Sciences*, *105*(10), 3921-3926.

[143] Oka, S. I., Liu, W., Masutani, H., Hirata, H., Shinkai, Y., Yamada, S. I., ... & Yodoi, J. (2006). Impaired fatty acid utilization in thioredoxin binding protein-2 (TBP-2)-deficient mice: a unique animal model of Reye syndrome. *The FASEB journal*, *20*(1), 121-123.

[144] Hui, T. Y., Sheth, S. S., Diffley, J. M., Potter, D. W., Lusis, A. J., Attie, A. D., & Davis, R. A. (2004). Mice lacking thioredoxin interacting protein provide evidence linking cellular redox state to appropriate response to nutritional signals. *Journal of Biological Chemistry*.

[145] Paulsen, C. E., & Carroll, K. S. (2013). Cysteine-mediated redox signaling: chemistry, biology, and tools for discovery. *Chemical reviews*, *113*(7), 4633-4679.

[146] Madan, E., Gogna, R., Kuppusamy, P., Bhatt, M., Mahdi, A. A., & Pati, U. (2013). SCO2 induces p53-mediated apoptosis by Thr845 phosphorylation of ASK-1 and dissociation of ASK-1-TRX complex. *Molecular and cellular biology*, MCB-06798.

[147] Zschauer, T. C., Kunze, K., Jakob, S., Haendeler, J., & Altschmied, J. (2011).

Oxidative Stress–Induced Degradation of Thioredoxin-1 and Apoptosis Is Inhibited by Thioredoxin-1–Actin Interaction in Endothelial Cells. *Arteriosclerosis, thrombosis, and vascular biology*, *31*(3), 650-656.

[148] Al-Gayyar, M. M., Abdelsaid, M. A., Matragoon, S., Pillai, B. A., & El-Remessy, A. B. (2011). Thioredoxin interacting protein is a novel mediator of retinal inflammation and neurotoxicity. *British journal of pharmacology*, *164*(1), 170-180.

[149] Zhou, R., Tardivel, A., Thorens, B., Choi, I., & Tschopp, J. (2010). Thioredoxin-interacting protein links oxidative stress to inflammasome activation. *Nature immunology*, *11*(2), 136.

[150] Johnson-Wood, K., Lee, M., Motter, R., Hu, K., Gordon, G., Barbour, R., ... & Lieberburg, I. (1997). Amyloid precursor protein processing and Aβ42 deposition in a transgenic mouse model of Alzheimer disease. *Proceedings of the National Academy of Sciences*, *94*(4), 1550-1555.

[151] O'Brien, R. J., & Wong, P. C. (2011). Amyloid precursor protein processing and Alzheimer's disease. *Annual review of neuroscience*, *34*, 185-204.

[152] de Silva, H. R., Jen, A., Wickenden, C., Jen, L. S., Wilkinson, S. L., & Patel, A. J. (1997). Cell-specific expression of β-amyloid precursor protein isoform mRNAs and proteins in neurons and astrocytes. *Molecular brain research*, *47*(1-2), 147-156.

[153] Hardy, J. A., & Higgins, G. A. (1992). Alzheimer's disease: the amyloid cascade hypothesis. *Science*, *256*(5054), 184.

[154] Zhang, Y. W., Thompson, R., Zhang, H., & Xu, H. (2011). APP processing in Alzheimer's disease. *Molecular brain*, *4*(1), 3.

[155] Sisodia, S. S. (1992). Beta-amyloid precursor protein cleavage by a membrane-bound protease. *Proceedings of the National Academy of Sciences*, *89*(13), 6075-6079.

[156] De Strooper, B., Iwatsubo, T., & Wolfe, M. S. (2011). Presenilins and γ-secretase: structure, function, and role in Alzheimer disease. *Cold Spring Harbor perspectives in medicine*, a006304.

[157] Haass, C., Kaether, C., Thinakaran, G., & Sisodia, S. (2012). Trafficking and proteolytic processing of APP. *Cold Spring Harbor perspectives in medicine*, a006270.

[158] Mattson, M. P. (1997). Cellular actions of beta-amyloid precursor protein and its soluble and fibrillogenic derivatives. *Physiological reviews*, *77*(4), 1081-1132.

[159] Kim, H. S., Kim, E. M., Lee, J. P., Park, C. H., Kim, S., Seo, J. H., ... & Suh, Y. H. (2003). C-terminal fragments of amyloid precursor protein exert neurotoxicity by inducing glycogen synthase kinase-3β expression. *The FASEB Journal*, *17*(13), 1951-1953.

[160] Vassar, R., Kovacs, D. M., Yan, R., & Wong, P. C. (2009). The β-secretase enzyme

BACE in health and Alzheimer's disease: regulation, cell biology, function, and therapeutic potential. *Journal of Neuroscience*, *29*(41), 12787-12794.

[161] Habib, A., Sawmiller, D., & Tan, J. (2017). Restoring Soluble Amyloid Precursor Protein α Functions as a Potential Treatment for A lzheimer's Disease. *Journal of neuroscience research*, *95*(4), 973-991.

[162] Murphy, M. P., & LeVine III, H. (2010). Alzheimer's disease and the amyloid-β peptide. *Journal of Alzheimer's Disease*, *19*(1), 311-323.

[163] Li, Y., Sun, H., Chen, Z., Xu, H., Bu, G., & Zheng, H. (2016). Implications of GABAergic neurotransmission in Alzheimer's disease. *Frontiers in aging neuroscience*, *8*, 31.

[164] Snyder, E. M., Nong, Y., Almeida, C. G., Paul, S., Moran, T., Choi, E. Y., ... & Greengard, P. (2005). Regulation of NMDA receptor trafficking by amyloid-β. *Nature neuroscience*, *8*(8), 1051.

[165] Apel, K., & Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.*, *55*, 373-399.

[166] Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of physiology*, *552*(2), 335-344.

[167] Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal*, *417*(1), 1-13.

[168] Simon, H. U., Haj-Yehia, A., & Levi-Schaffer, F. (2000). Role of reactive oxygen species (ROS) in apoptosis induction. *Apoptosis*, *5*(5), 415-418.

[169] Halliwell, B. (1992). Reactive oxygen species and the central nervous system. *Journal of neurochemistry*, *59*(5), 1609-1623.

[170] Fubini, B., & Hubbard, A. (2003). Reactive oxygen species (ROS) and reactive nitrogen species (RNS) generation by silica in inflammation and fibrosis. *Free Radical Biology and Medicine*, *34*(12), 1507-1516.

[171] Van Der Vliet, A., Eiserich, J. P., Halliwell, B., & Cross, C. E. (1997). Formation of reactive nitrogen species during peroxidase-catalyzed oxidation of nitrite a potential additional mechanism of nitric oxide-dependent toxicity. *Journal of Biological Chemistry*, *272*(12), 7617-7625.

[172] Emerit, J., Edeas, M., & Bricaire, F. (2004). Neurodegenerative diseases and oxidative stress. *Biomedicine & pharmacotherapy*, *58*(1), 39-46.

[173] Jan, A., Hartley, D. M., & Lashuel, H. A. (2010). Preparation and characterization of toxic Aβ aggregates for structural and functional studies in Alzheimer's disease research. *Nature protocols*, *5*(6), 1186.

[174] Kayed, R., Head, E., Thompson, J. L., McIntire, T. M., Milton, S. C., Cotman, C. W., & Glabe, C. G. (2003). Common structure of soluble amyloid oligomers implies common mechanism of pathogenesis. *Science*, *300*(5618), 486-489.

[175] Maezawa, I., Hong, H. S., Liu, R., Wu, C. Y., Cheng, R. H., Kung, M. P., ... & Jin, L. W. (2008). Congo red and thioflavin-T analogs detect Aβ oligomers. *Journal of neurochemistry*, *104*(2), 457-468.

[176] Gould, N., Doulias, P. T., Tenopoulou, M., Raju, K., & Ischiropoulos, H. (2013). Regulation of protein function and signaling by reversible cysteine S-nitrosylation. *Journal of Biological Chemistry*, jbc-R113.

[177] Chouchani, E. T., Kazak, L., Jedrychowski, M. P., Lu, G. Z., Erickson, B. K., Szpyt, J., ... & Robinson, A. J. (2016). Mitochondrial ROS regulate thermogenic energy expenditure and sulfenylation of UCP1. *Nature*, *532*(7597), 112.