PRP\textsuperscript{C} AND CELL CYCLE RE-ENTRY: A NOVEL PATHOGENIC MECHANISM FOR Aβ NEUROTOXICITY

By

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Dedication

To my parents, my friends, and family members. Without their patience and compassion, I would not have completed my Ph.D study.
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List of Abbreviations

AD: Alzheimer’s disease

AβPP: Amyloid-β precursor protein

Aβ: Amyloid β

APV: (2R)-amino-5-phosphonopentanoate

ATM: Ataxia telangiectasia mutated

AMPA: 2-amino-5-hydroxy- 3-methyl-4-isoxazole propionate

Bcl-2: B-cell lymphoma 2

BrdU: 5’-bromodeoxyuridine

BIP: Bax-inhibiting peptide

CaMKII: Ca\(^{2+}\)/calmodulin-dependent protein kinase

CDK: Cyclin-dependent kinase

DAPI: 4’, 6-diamidino-2-phenylindole

DG: Dentate gyrus

E2F: E2 promoter transcription factor

ERK1/2: extracellular signal-regulated protein kinase

FITC: Fluorescein isothiocyanate
γH2AX: Gamma histone H2A

GPCRs: G-protein-coupled receptors

GPI: Glycosylphosphatidylinositol

H&E: Hematoxylin and Eosin-Y

HBSS: Hank's buffered salt solution

IP: Immunoprecipitation

JNK: c-Jun N-terminal kinase

KO: Knockout

LTP: Long-term potentiation

MAPK: mitogen-activated protein kinase

MCI: Mild cognitive impairment

mGluR5: Metabotropic glutamate receptor 5

NFTs: Neurofibrillary tangles

NMDA: N-methyl-D-aspartate

NOS: Nitric oxide synthase

NR2B: NMDA receptor subunit 2B

PCNA: Proliferating cell nuclear antigen
PHF: Paired helical filaments

PI: Propidium Iodide

PLA2: Phospholipase A2

PrP: Prion protein

PSD-95: Postsynaptic density protein 95

PVDF: Polyvinylidene fluoride

Rb: Retinoblastoma protein

SFK: Src family kinases

TUNEL: Terminal dUTP Nick-End Labeling

WT: Wild type
Alzheimer's disease (AD), a progressive neurodegenerative disorder, is the most common cause of dementia in the elderly. Characteristic features in AD include neurofibrillary tangles, neuronal cell loss and extracellular deposits of the amyloid β (Aβ) protein. Although, among Aβ species, oligomeric Aβ is the most neurotoxic, the mechanisms through which Aβ causes neuronal death are still unclear.

A role for the cellular prion protein (PrP<sup>C</sup>) in mediating the neurotoxic effect of oligomeric Aβ was proposed although several subsequent studies presented apparently contradictory results. In Chapter 2, I show that Prnp<sup>-/-</sup> mice are more resistant to the neurotoxic effect of oligomeric Aβ than wild type mice using both in vivo and in vitro assays. Furthermore, application of anti-PrP antibody or PrP peptide rescues oligomeric Aβ-induced neurotoxicity in a hippocampal slice culture model system. Next, as the crux of my thesis work, I investigated the signaling mechanisms by which oligomeric Aβ induces neurotoxicity through
PrP\textsuperscript{C}. Presented in Chapter 3, oligomeric Aβ was found to induce N-methyl-D-aspartate (NMDA) receptor subunit 2B (NR2B) phosphorylation through Fyn activation in a manner dependent on PrP\textsuperscript{C} expression. To complete the mechanistic pathway, caveolin-1 was determined to play a critical role in the phosphorylation of NR2B and neurotoxicity. Taken together, these data indeed demonstrate that caveolin-1 acts as a scaffold protein for Fyn and PrP\textsuperscript{C}, and is critical in oligomeric Aβ-induced neurotoxic signaling.

We showed that dysregulation of cell cycle re-entry results in neurodegeneration in a transgenic mouse model; however, the molecular mechanisms causing neuronal cell cycle re-entry in AD remain unknown. In Chapter 4, the central role of cell cycle re-entry in Aβ-mediated neuronal cell death is established and that the degradation of p27\textsuperscript{Kip} through CaMKII-ERK1/2 signaling is identified as the key pathway for promoting neuronal cell cycle progression and cell death. Finally, in Chapter 5, I demonstrate that Bax, a pro-apoptotic protein, plays an essential role in oligomeric Aβ-induced neuronal cell death.

Overall, my findings indicate that PrP\textsuperscript{C} and caveolin-1 play central roles in initiating neurotoxicity by oligomeric Aβ, and that oligomeric Aβ induces cell cycle re-entry in neurons through the CaMKII-ERK1/2 signaling pathway, leading to neuronal death.
CHAPTER 1: Background and Specific Aims
BACKGROUND:

Alzheimer’s disease

Alzheimer’s disease (AD) is the leading cause of dementia in individuals over the age of sixty five or older. As many as 350,000 more individuals develop the disease each year and current estimates speculate that by 2050 over 14 million Americans will have AD [1]. An estimated 4 million individuals in the United States have AD with an annual cost of $70-$100 billion. Characterized clinically by progressive memory loss, cognitive decline and eventual loss of motor skills, the disease is only definitively diagnosed after post-mortem demonstration of the neuropathological hallmark of the disease, senile plaques and neurofibrillary tangles. The parts of the brain that control thought, memory, and language are affected in AD. There are several major theories for the cause of AD such as Aβ toxicity [2], inflammation [3], and oxidative stress [4]. Evidence from genetic, cell biological and postmortem studies on AD brains suggests that extensive deposits of Aβ play a critical role in the pathogenesis of the disease [5]. However the mechanisms underlying the neurodegeneration remain unclear.

Pathology in Alzheimer’s disease

The hallmarks of AD are two distinctive lesions in the patient’s brain, senile plaques and neurofibrillary tangles (NFTs) [6]. They were identified by silver staining of the AD patient’s brains. In addition, other pathological changes are
detected in the AD patient’s brains, such as neuronal and dendritic loss, neuropil threads and dystrophic neurites [7]. Senile plaques are spherical extracellular lesions made up of 6-10 nm bundles of Aβ protein [7]. In the peripheral region of the senile plaques, amyloid β (Aβ), amyloid-β precursor protein (AβPP), τ, and neurofilament proteins are observed [8]. NFTs are major intracellular protein aggregates found in AD brains. They are located primarily in the cerebral cortex, especially in the large pyramidal neurons in the hippocampal and frontotemporal regions [7]. NFTs are composed of bundles of paired helical filaments (PHF), the major component of which is the microtubule-associated protein τ.

**Amyloid β**

Aβ is the major component of senile plaque cores and it is derived from a precursor protein called AβPP. AβPP can be processed by α-, β- and γ-secretases. Aβ protein is generated by successive action of the β- and γ-secretases. The γ-secretases, which produce the C-terminal end of the Aβ peptide, cleaves within the transmembrane region of AβPP and can generate a number of isoforms of 39-43 amino acid residues in length. The most common isoforms are Aβ1-40 and Aβ1-42. The Aβ1-40 form is the more common of the two, but Aβ1-42 is the more fibrillogenic and is thus associated with disease states. Mutation in AβPP associated with early-onset AD have been noted to increase the relative production of Aβ1-42, and thus it is suggested that therapeutic target for AD involves modulating the activity of β- and γ-secretases. The mature fibril Aβ has
been believed to have toxic effect on neurons. In light of the fact that the degree of insoluble Aβ deposition in the brain correlates poorly with cognitive decline, investigators have turned their attention to soluble aggregates of Aβ, called oligomeric Aβ. Several studies have suggested that oligomeric forms of Aβ are acutely toxic and may relate to episodic memory deficits, synapse degeneration and nerve cell death [9, 10]. The pathogenic relevance of this form of Aβ has been substantiated by a newly identified Arctic familial AD mutation, which has an increased propensity to oligomerize [11], and by *in vitro* data demonstrating potent neurotoxicity upon exposure to soluble oligomers or protofibrils [12, 13]. *In vivo* data showed that intrahippocampal injection of Aβ1–42 rats showed remarkable memory impairment in Morris water maze test and neuronal apoptosis [14]. furthermore, the injection of oligomeric Aβ disrupts learned behavior in a manner that was rapid, potent and transient [15]. These findings tell us that oligomeric Aβ is sufficient to cause behavior impairment and cognitive dysfunction. However, the signaling mechanism(s) inducing the dysfunction *ex vivo* and *in vivo* model remains unclear.
Figure 1-1. AβPP processing. Amyloid-β precursor protein (AβPP) is a transmembrane protein that can undergo a series of proteolytic cleavage by secretase enzymes. When it is cleaved by α-secretase in the middle of the Aβ, it is not amyloidogenic. However, when APP is cleaved by β- and γ-secretase enzymes, neurotoxic Aβ peptides are released, which can accumulate into oligomer aggregate.

PrP<sup>C</sup>; A potential receptor for Aβ

Several receptor proteins have been proposed to be capable of binding various forms of Aβ, thereby inducing its cellular effects. These now include advanced glycation end product [16], NMDA [17], metabotrophic glutamate receptor 5 (mGluR5) [18] and insulin receptors [19]. Recently, a role for the cellular prion protein (PrP<sup>C</sup>) in the neurotoxic effect of oligomeric Aβ was proposed, although several subsequent studies presented apparent contrasting results. Initially, Lauren et al. reported the specific binding of oligomeric Aβ to PrP<sup>C</sup>, and that this interaction was essential for oligomeric Aβ-induced synaptic toxicity as measured
by analysis of long-term potentiation (LTP) [20]. Subsequently, they demonstrated that the ablation of PrP<sup>C</sup> enhances cognitive function in transgenic mice overexpressing mutant APP genes linked to familial AD (APPswe and PS1ΔE9) and prevents early death and memory impairment [21]. However, several studies by other groups reported that the lack of PrP<sup>C</sup> did not prevent oligomeric Aβ-mediated synaptic toxicity and cognitive impairment [22-24]. Thus, while our study, along with others, confirms the physical interaction between Aβ and PrP<sup>C</sup>, it is controversial whether the binding of Aβ and PrP<sup>C</sup> is required for neurotoxicity of oligomeric Aβ.

**Caveolae and Caveolin-1**

Caveolae are invaginations of the plasma membrane containing caveolin proteins (caveolin-1, -2, and -3) and are considered a subset of lipid rafts. These invaginations originally named “plasmalemmal vesicles” are small (50-100 nanometer) and occur in most cell types including endothelial cells. While the structure is typically flask-shaped, other morphologies such as flat, tubular, and vesicular are noted. Caveolae are considered to be a highly curved subset of lipid rafts that contain the integral membrane protein caveolin-1 and are rich in cholesterol, sphingomyelin, glycosphingolipids, and signaling molecules [25].

Caveolin-1 is the main structural protein of caveolae, serving as the caveolae backbone. Both termini of caveolin-1 are exposed in the cytoplasm (Figure 1-2), which results from formation of a hairpin loop in the cytoplasmic leaflet of the
plasma membrane by a central intermembrane domain (aa 97-131). The protein contains a scaffolding domain (CSD) of ~20 amino acids (residues 82–101 for canine caveolin-1, 54–73 for human caveolin-2 and 55–74 for rat caveolin-3) that facilitates interaction with numerous other proteins, including Src family tyrosine kinases, growth factor receptors, G-protein-coupled receptors (GPCRs), G proteins and endothelial nitric oxide synthase (NOS) [26-28]. Evidence for these interactions for the signaling components led to a ‘caveolin signaling hypothesis’, which posits that caveolins concentrate and regulate signal transduction molecules by virtue of the ability of the CSD to bind signaling components.

High levels of caveolin are expressed in several cell types, but such expression does not necessarily equate to caveolae. For example, cardiac myocytes express substantial amounts of all three caveolin isoforms although they have fewer caveolae than those other cell types [29], suggesting that there are caveolins outside plasma membrane caveolae. Recent studies indicate that caveolin can contribute to cell regulation in regions other than caveolae: such regulation includes signaling proteins involved in cAMP production [30], cell adhesion [31], injury-induced neuronal plasticity [32]. Furthermore, neurons express all three caveolins [32, 33] but lack caveolae [34, 35], even though detergent-resistant, low-density membranes (lipid rafts) can be isolated from these cells. These caveolins are functional, perhaps serving as scaffolds for signaling complexes involved in neuronal transmission and plasticity, such as in the actions of the neurotransmitter glutamate: caveolin-1 interacts with glutamate receptors and through its CSD can modify the ionotropic receptor for glutamate
and 2-amino-5-hydroxy-3-methyl-4-isoxazole propionate (AMPA) by regulating phospholipase A2 (PLA2) activity [36]. Importantly, neuronal caveolin-1 can interact with GPI-anchored PrPc and synaptophysin, a protein involved in exocytosis and neurotransmission, in neuronal DRMs [37]. Thus, neuronal caveolin seems to be involved in the regulation of neurotrophin signal transduction as well as in neurite sprouting and synaptogenesis.

![Figure 1-2. A model of caveolin isoforms (caveolin-1) insertion into the plasma membrane.](image)

The predicted membrane topology of caveolin-1. About 14-16 monomers normally self-associate to form a single caveolin homo-oligomer (the caveolar assembly unit, akin to the clathrin triskelion). Note that both the amino- and carboxy-terminal domains are oriented towards the cytosolic face of the plasma membrane, with a hairpin loop structure inserted within the membrane bilayer.

**Cell Cycle in Eukaryotic Cells**

The eukaryotic cell cycle is a series of events that lead to cell growth, DNA replication, chromosomal segregation and a creation of two daughter cells from
one original cell. These events are divided into four functionally distinct phases: Growth 1 phase (G1), DNA synthesis (S), Growth 2 phase (G2) and Mitosis (M). G1 is a period of growth and preparation for division. S phase follows G1 and is marked by replication of the genetic material in the cell. After the duplication, the cell prepares for division in the G2 phase. After chromosome condensation and movement to the opposite poles of the cell, the cytoplasm splits and cell division occurs, culminating in the generation of two new cells. In vertebrates, the initiation and progression of the cell cycle is regulated by a family of cyclin dependent kinases (Cdks) formed by a catalytic subunit and a regulatory subunit termed cyclin family. These kinases act sequentially during the cell cycle. Specific cyclins/kinases are expressed and active during each phase of the cycle; Cdk4 or Cdk6 pair with cyclin D during G1 and the G1/S transition, Cdk2 and cyclin A are active during S phase and cdc2/cyclin B exert their activity during M phase.

Adding further complexity and regulation to this system are cell cycle inhibitors that block cdk/cyclin activity by either forming an inactive complex or by acting as a competitive Cdk ligand. Members of the INK family, such as p19INK4d and p15INK4b, inhibit Cdk4(6)/cyclin D. The cyclin A/Cdk2 complex is inhibited by the Cip/Kip family members. In addition to Cdks and cyclins during the cell cycle, several other proteins play a role in regulating cell cycle initiation and progression. For example, E2F/DP1 protein pair is a well-studied member of the cell cycle. In non-dividing cells the E2F family of proteins is bound to retinoblastoma protein (RB) and act as a transcriptional repressor.
Malfunction of neuronal cell cycle in AD

Accumulating evidence suggests that aberrant neuronal cell cycle re-entry may precede the regional neurodegeneration observed in AD [38-40]. In mature neurons, the cell cycle is normally arrested at the G_0 phase, but neurons vulnerable to degeneration in AD are phenotypically suggestive of cells that have re-entered into the cell cycle. Notably, while various mitotic markers are upregulated in vulnerable neurons in AD, no evidence exists of actual mitosis [41]. This suggests that these neurons are arrested at a point(s) prior to the physical event of cellular division, and once cyclin A is expressed the arrested cells lack the ability to return to G_0 and therefore must either complete the cycle or die. Correspondingly, we recently showed that dysregulation of cell cycle re-entry results in neurodegeneration in vivo using MYC transgenic mice [42]. From these data, it has been thought that the activation of cell cycle processes is part of a mechanism by which loss of tropic support during development leads to neuronal cell death in AD. While several studies have reported the correlation between AD and cell cycle re-entry, there is a lack of evidence about the exact signaling events associated with cell cycle re-entry occurs in AD.

Recent studies have demonstrated that oligomeric Aβ, but not monomeric Aβ, can induce neuronal cell cycle induction in primary cortical neurons [43]. However, few studies have tried to understand how the oligomeric Aβ affect neuronal cell cycle and how the oligomeric Aβ may modulate this process at the molecular level. Furthermore, there is little known about the precise role of the oligomeric Aβ in the modulation of neuronal loss and the molecular basis
associated with neurotoxicity in response to this molecule in the hippocampus. In
addition to the example given for neuronal cell cycle in AD, there is also evidence
that neuronal cell cycle re-entry occurs early in the disease process. For example,
immunohistochemical studies demonstrate that individuals with mild cognitive
impairment (MCI), widely viewed as the clinical predecessor to AD, also exhibit
neuronal cell cycle re-entry [44]. The presence of neuronal cell cycle re-entry in
MCI suggests that neuronal cell cycle re-entry is not limited to the final stages of
the AD; rather, they are associated with brain regions subject to degeneration
throughout the entire period of disease.

SPECIFIC AIMS:

I worked on four main projects to clarify the mechanism(s) by which
oligomeric Aβ induces neurotoxicity.

*Cellular Prion Protein Is Essential for Oligomeric Amyloid-β-Induced
Neuronal Cell Death (Chapter 2)*; I found that PrpC is required for oligomeric
Aβ-induced neuronal cell death, the pathology essential to cognitive loss.

*Caveolin-1 Mediate Oligomeric Amyloid β-Induced Neurotoxic Signaling
Through Cellular Prion Protein (Chapter 3)*; I demonstrated that caveolin-1
mediates oligomeric Aβ-induced neurotoxic signaling through PrP$_{C}^{C}$. 
Toxicity of Soluble Amyloid Oligomers through Neuronal Cell Cycle Re-entry (Chapter 4): I demonstrated the central role of cell cycle re-entry in oligomeric Aβ mediated neuronal cell death and that the degradation of p27^Kip through CaMKII-ERK1/2 signaling is the key mechanism for promoting neuronal cell cycle progression.

Inhibition of Bax Protects Neuronal Cells from Oligomeric Aβ-induced Toxicity (Chapter 5): I showed that Bax plays an important role in oligomeric Aβ-induced neuronal cell death, and that targeting Bax may provide a therapeutic strategy for treating Alzheimer’s disease.
CHAPTER 2: Cellular Prion Protein Is Essential for Oligomeric Amyloid-β-Induced Neuronal Cell Death
Abstract

In Alzheimer disease (AD), oligomeric amyloid-β (Aβ) is suggested to play a critical role in imitating neurodegeneration although its pathogenic mechanism remains to be determined. Recently, the cellular prion protein (PrP^C) has been reported to be an essential co-factor in mediating the neurotoxic effect of oligomeric Aβ. However, these previous studies focused on the synaptic plasticity in either the presence or absence of PrP^C and no study to date has reported whether PrP^C is required for the neuronal cell death, the most critical element of neurodegeneration in AD. Here, I show that Prnp^−/− mice are resistant to the neurotoxic effect of oligomeric Aβ in vivo and in vitro. Furthermore, application of an anti-PrP^C antibody or PrP^C peptide prevents oligomeric Aβ-induced neurotoxicity. These findings are the first to demonstrate that PrP^C is required for oligomeric Aβ-induced neuronal cell death, the pathology essential to cognitive loss.

Introduction

Recently, it has been demonstrated that the specific binding of oligomeric amyloid-β (Aβ) to cellular prion protein (PrP^C) is essential for synaptic toxicity reflected in loss of long-term potentiation (LTP) [20]. Moreover, ablation of PrP^C enhances cognitive function in transgenic mice overexpressing mutant amyloid precursor protein (APP) genes (APPswe and PS1ΔE9) preventing premature death and memory impairment [21]. However, other reports questioned these findings
by noting that lack of PrP<sub>C</sub> did not prevent Aβ oligomer-mediated synaptic toxicity or cognitive impairment [22-24]. Thus, while our recent study [45], along with others, confirms the physical interaction between Aβ and PrP<sub>C</sub>, it remains unclear whether PrP<sub>C</sub> is essential to neurotoxicity of oligomeric Aβ.

The apparent conflict may be due to the assays used, for previous reports all focused on synaptic plasticity [20, 22, 24, 46, 47], rather than neuronal cell death, the final pathway defining Alzheimer disease (AD). Here, I report that neuronal cell death induced by synthetic oligomeric Aβ was prevented by reducing or eliminating PrP<sub>C</sub>, or blocking the binding between PrP<sub>C</sub> and oligomeric Aβ using either a PrP<sub>C</sub> antibody or a decoy PrP<sub>C</sub> peptide. These findings strongly suggest that PrP<sub>C</sub> is required for Aβ induced neuronal cell death. This is the first demonstration that the PrP<sub>C</sub>/Aβ interaction is necessary for neuronal cell loss, the pathology underlying cognitive decline in AD.

**Materials and Methods**

**Mouse strains**

*Prnp<sup>−/−</sup>* mice (Zürich1) [48] backcrossed onto the FVB/N background were obtained from George Carlson, McLaughlin Research Institute, Great Falls, Montana.

*Preparation of oligomeric Aβ*
Soluble oligomeric Aβ was prepared from synthetic peptide as described previously [49].

Preparation of hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as described [50]. Briefly, hippocampal slice cultures were prepared from 7-10 day-old mouse pups. 400 μm slices were cut using a McIlwain tissue chopper and transferred to Millicell (Millipore Corp.) membrane inserts (0.4 μm).

Assessment of neuronal cell death

To determine neuronal cell death in the hippocampal slices, PI was added to the slice culture medium. Images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss). Nissl staining was also performed for routine histochemical and morphological analyses. TUNEL staining followed a previous protocol [42]. Western blots for detection of caspase-3 were performed as previously described [51].

Intrahippocampal injection of oligomeric Aβ

FVB mice (2-3 month old; Jackson Laboratories) were anaesthetized with pentobarbital and placed in a stereotaxic frame. Injection was made using a 10 μl
microsyringe (Hamilton). 1 μl of oligomeric Aβ, 50 μM in PBS, was injected into the left hippocampus. Control animals were prepared identically and injected with the same concentration of Aβ_{42-1} in PBS (reversed sequence of Aβ_{1-42}). Injections were made at stereotaxic coordinates of Bregma; anteroposterior (AP) = 2.3mm, mediolateral (ML) = 2.5mm, dorsoventral (DV) = -2.5mm. This corresponds to a site in the dorsal hippocampus in the apical dendritic zones of the CA1 region near the hippocampal fissure. Mice were sacrificed 20 days after injection, brains dissected out, fixed in 10% buffered formalin and paraffin embedded.

**Statistical analysis**

Data were expressed as the means ± S.E.; the number of independent experiments is indicated in the corresponding figure legend. Differences between groups were examined for statistical significance using one-way analysis of variance with an unpaired Students t-test. A \( p \) value less than 0.05 indicated a statistically significant difference.

**Results**

**PrP\textsuperscript{C} is required for oligomeric Aβ induced neurotoxicity in slice culture.**

I first examined the effect of oligomeric Aβ on neuronal cell death in hippocampal slice cultures prepared from wild type (WT) or \( Prnp^{−/−} \) mice (Figures 2-1a and b) to test whether PrP\textsuperscript{C} is necessary for oligomeric Aβ-mediated
neuronal cell death. Consistent with previous studies [52], oligomeric Aβ (500 nM) induced significant neuronal cell death in WT samples, as measured by propidium iodide (PI) staining (Figure 2-1c). However, the neuronal cell death induced by oligomeric Aβ was dramatically decreased in slice cultures prepared from Prnp⁻/⁻ mice (Figure 2-1c). This result was confirmed by TUNEL assay which demonstrated the increase of cell death by oligomeric Aβ in WT mice but not in Prnp⁻/⁻ mice (Figure 2-1d). In addition, activation of caspase-3 after oligomeric Aβ treatment was attenuated in slices from Prnp⁻/⁻ mice (Figure 2-1e). Thus, PrP^C is required for oligomeric Aβ-induced neurotoxicity.
Figure 2-1. PrP<sup>C</sup> is essential for Aβ oligomer-induced neurotoxicity. a) DNA was extracted from WT and Prnp<sup>−/−</sup> mice and each genotype was identified by PCR with the primer sets specifically detecting each genotype as described in the previous study [48]. WT yields an 1100 bp PCR product and Prnp<sup>−/−</sup> yields an 850 bp PCR product. b) Total protein (20 μg) from whole brain of the indicated genotype was analyzed by immunoblot with anti-PrP antibody (6D11). Immunoblot analysis shows the expected glycoforms of PrP<sup>C</sup> in WT samples migrating between 25–37 kDa, and no band in Prnp<sup>−/−</sup> samples. c) The slices were treated with Aβ<sub>1-42</sub> oligomer or the control reverse Aβ<sub>42-1</sub> peptide (500 nM) in the presence of PI (red) for 48 h. Representative pictures showed Aβ oligomer-induced PI staining was significantly reduced in the slice cultures from Prnp<sup>−/−</sup> mice. The PI staining was quantitatively analyzed (n=5). Slices from wild type (WT) or Prnp<sup>−/−</sup> mice with the control Aβ<sub>42-1</sub> peptide show no difference. Scale bar, 500 μm. (**p<0.01 or *p<0.05). d) The number of TUNEL positive cells (green) was also significantly reduced in Prnp<sup>−/−</sup> hippocampal slice culture (n=5). Scale bar, 100 μm (*p<0.05). e) Representative western blot data shows that the expression of active caspase-3 is significantly reduced in Prnp<sup>−/−</sup> slices after a 24 h treatment with Aβ oligomer (n=4) (***p<0.01 or *p<0.05).
Blocking the PrP<sup>C</sup>-oligomeric Aβ interaction inhibits neurotoxicity.

To block the PrP<sup>C</sup>-oligomeric Aβ interaction, I treated the slice cultures with the anti-PrP<sup>C</sup><sub>93-109</sub> antibody 6D11 (10 µg/ml), which binds to the critical region of PrP<sup>C</sup>/Aβ binding (amino acids 93-109), and examined whether the antibody could prevent neuronal cell death induced by oligomeric Aβ. A previous report showed that 6D11 antibody blocked the binding of oligomeric Aβ to PrP<sup>C</sup> [20], while another anti-PrP<sup>C</sup> antibody, 6H4, which recognizes PrP<sup>C</sup><sub>144-152</sub>, failed to block oligomeric Aβ/PrP<sup>C</sup> binding. In the hippocampal slice cultures prepared from WT mice, pretreatment with 6D11 significantly reduced PI staining and caspase-3 activation following treatment with oligomeric Aβ, while pretreatment with normal mouse IgG or 6H4 antibody failed to reduce either marker of cell death (Figure 2-2a and b). I extended the competitive inhibition approach by using specific PrP<sup>C</sup> peptides either containing the Aβ binding region or not [45]. Consistent with the antibody blocking experiment, addition of the peptide corresponding to PrP<sup>C</sup><sub>98-107</sub> (Pep29, 500 nM) dramatically reduced the neurotoxicity of oligomeric Aβ in the hippocampal slice cultures (Figure 2-2c and d). In contrast, the peptide corresponding to PrP<sup>C</sup><sub>213-230</sub> (Pep56) had no effect on oligomeric Aβ-induced neurotoxicity (Figure 2-2c and d). Further, addition of either peptide without oligomeric Aβ treatment had no effect on cell death (Figure 2-2c and d). Nissl staining confirmed that co-application of the peptide corresponding to PrP<sup>C</sup><sub>98-107</sub> with oligomeric Aβ prevented neuronal loss (data not shown). I also tested whether a caspase-inhibitor (Z-VAD-FMK) can prevent the cell death and found that the caspase inhibitor significantly prevented neuronal
cell death (Figure 2-3a) corresponding to the level of caspase-3 activation (Figure 2-3b). These results confirmed that PrP^C/Aβ binding is necessary for oligomeric Aβ mediated neurotoxicity.

In the previous studies, the link between PrP^C and NMDA receptor has been demonstrated [53, 54], suggesting the potential role of PrP^C in NMDA receptor mediated excitotoxicity. Therefore, I tested whether the inhibition of NMDA receptor can prevent neuronal cell death in my experimental model. Indeed, (2R)-amino-5-phosphonovaleric acid (APV), a NMDA antagonist, significantly reduced the level of neuronal cell death induced by oligomeric Aβ (Figure 2-3a).
Figure 2-2. Aβ oligomer-induced neuronal cell loss is prevented by blocking the PrPC-Aβ interaction with PrP\textsuperscript{C} specific antibodies or peptides. a) The intensity of PI in slices treated with Aβ oligomer after the addition of 6D11 antibody, control immunoglobulin G (IgG), or 6H4 1 h before Aβ oligomer treatment (n=5). The PI staining is significantly reduced by 6D11 antibody but not by either IgG or 6H4 antibody. Scale bar, 500 μm (**p<0.01 versus control #p<0.05 versus +IgG). b) Pretreatment with 6D11 suppressed the activation of caspase-3 induced by Aβ oligomer (n=4) (**p<0.01 versus control #p<0.05 versus +IgG). c) Co-treatment of synthesized peptide-29, corresponding to PrP\textsuperscript{C}\textsubscript{98-107}, significantly prevents Aβ oligomer-induced PI staining (n=5). Scale bar, 500 μm. (***p<0.01 versus control #p<0.05 versus non-peptide). d) Co-treatment with peptide-29 suppressed the activation of caspase-3 induced by Aβ oligomer (n=4) (**p<0.01 versus control #p<0.05 versus non-peptide).
Figure 2-3. Treatment with an NMDA antagonist (APV) or caspase inhibitor (Z-VAD-FMK) significantly attenuates Aβ oligomer induced PI staining. a) The slices were treated with Aβ1-42 oligomer (500 nM). APV (20 μM) or Z-VAD-FMK (Cas Inh) (20 μM) was co-applied with Aβ oligomer (n=5). Both APV and Z-VAD-FMK dramatically reduced the level of PI staining induced by Aβ1-42 oligomer. Scale bar, 500 μm (*p<0.05 versus Aβ). b) Pretreatment of caspase inhibitor, Z-VAD-FMK (20 μM), significantly reduced Aβ oligomer-induced caspase-3 activation (n=3) (**p<0.01 versus control, ##p<0.01 versus non-inhibitor).
**PrP<sup>C</sup> dependence of oligomeric Aβ–mediated neurotoxicity *in vivo***

I also established similar findings in an animal model. Intra-hippocampal injection of oligomeric Aβ has been shown to result in profound memory impairment and neuronal apoptosis *in vivo* [14]. I injected oligomeric Aβ into the hippocampus of either WT or *Prnp<sup>−/−</sup>* mice and analyzed neuronal cell death. Neuronal cell loss was evident in WT mice injected with oligomeric Aβ 20 days after injection. Importantly, consistent with the previous results in the hippocampal slice culture model, neuronal cell death was almost completely eliminated in *Prnp<sup>−/−</sup>* mice when compared to WT mice (Figure 2-4a and b) thus confirming the role of PrP<sup>C</sup> in oligomeric Aβ–induced neuronal cell death *in vivo*.

**Figure 2-4. PrP<sup>C</sup> is essential for Aβ oligomer–induced neurotoxicity *in vivo*.**

a) *Prnp<sup>−/−</sup>* and WT mice were sacrificed and brain tissues stained with H&E at 20 days after Aβ oligomer injection. Neuronal cell loss in hippocampus was evident in WT mice injected with Aβ oligomer (arrows) but not in *Prnp<sup>−/−</sup>* mice. Scale bar, 100 μm. b) The number of TUNEL positive cells (arrows) in hippocampus was dramatically reduced in *Prnp<sup>−/−</sup>* mice after Aβ oligomer injection compared to WT mice (n=5). Scale bar, 100 μm (*p<0.05).
Discussion

The present study strongly supports a critical role for PrP\textsuperscript{C} in mediating the neurotoxic effect of oligomeric Aβ. While previous research focused on the effect of oligomeric Aβ in synaptic impairment \textit{in vitro}, I provide additional convincing \textit{in vitro} and \textit{in vivo} evidences that the PrP\textsuperscript{C}/Aβ interaction is necessary for triggering neuronal cell loss. In a recent study supporting this view, Resenberger and colleagues demonstrated that overexpression of PrP\textsuperscript{C} in neuronal cell cultures increased vulnerability to the neurotoxic effects of various β-sheet-rich conformers, including Aβ [54]. My results provide further support for this conclusion based on the results obtained using a knockout of PrP\textsuperscript{C}, or blocking the PrP\textsuperscript{C}-Aβ interaction by the use of PrP\textsuperscript{C} specific antibodies or peptide under more physiological conditions (summarized in Figure 2-5).

The pretreatment with the antibody 6D11, which binds PrP\textsuperscript{C}_{93-109}, prevents neuronal cell death by oligomeric Aβ, while another anti-PrP\textsuperscript{C} antibody, 6H4, which recognizes PrP\textsuperscript{C}_{144-152}, failed to block oligomeric Aβ-induced neuronal toxicity. Furthermore, consistent with the antibody experiments, addition of the peptide corresponding to residues PrP\textsuperscript{C}_{98-107} reduced the neurotoxicity of oligomeric Aβ in the hippocampal slice cultures, whereas the peptide corresponding to residues PrP\textsuperscript{C}_{213-230} had no effect on the oligomeric Aβ-induced neurotoxicity. These data indicate it is the essential region PrP\textsuperscript{C}_{98-107} in PrP\textsuperscript{C} that mediates oligomeric Aβ-PrP\textsuperscript{C} interaction. This binding site is similar to the sequence (amino acids 95-105) identified in a previous study [20] which showed that the treatment with antibody binding this region prevented the interaction and
oligomeric Aβ -induced LTP [20] and improved cognitive deficits in aged AD transgenic mice [55]. More recent studies also confirmed that an anti-PrP antibody targeted to PrP\textsubscript{C\textsuperscript{93-102}} blocks LTP induced by Aβ-containing AD brain extract [46, 47]. Collectively, my results strongly suggest that PrP\textsubscript{C\textsuperscript{98-107}} contains the critical amino acid sequence for oligomeric Aβ-induced synaptic impairment and neuronal cell death.

While the current data support the pathological role of PrP\textsubscript{C}/Aβ interaction, three independent studies failed to confirm a critical role of PrP\textsubscript{C} in vivo and in vitro [22-24]. The exact reason for this discrepancy needs to be examined, but differences in the experimental conditions, such as the specific transgenic animal model or in the preparation of oligomeric Aβ might be possible reasons. Additionally, PrP\textsubscript{C} is apparently not the only cellular surface protein that interacts with oligomeric Aβ, since elimination of PrP\textsubscript{C} only reduces oligomeric Aβ binding by 50% to cultured hippocampal neurons [20]. Several putative receptor sites have been proposed to mediate neurotoxic signaling of oligomeric Aβ, such as the receptor of advanced glycation end product [16], NMDA [54], insulin [19] and p75 neurotrophin receptor [56]. Consistent with this result, my data showed that blocking of PrP\textsubscript{C}/Aβ interaction, either by application of an anti-PrP antibody or competitive peptides, inhibits ~60% of oligomeric Aβ -induced neuronal cell loss. These results further support the idea that other neurotoxic signaling pathways, which are independent of PrP\textsubscript{C}, may contribute to neurotoxicity.

A previous report suggested that NMDA receptor-mediated excitotoxicity might be the downstream mechanism of Aβ neurotoxicity [54], which was also
confirmed in my study. Although further studies will be required to elucidate the pathological mechanism(s) in detail, a mechanistic link between Aβ-PrP\(^C\) and the NMDA receptor for neurotoxicity is further supported by the previous finding that an NMDA antagonist prevents Aβ-induced neuronal loss and disruption of synaptic plasticity [57]. In addition, oligomeric Aβ was found to directly or indirectly bind NMDA receptor [18] and PrP\(^C\) is also reported to interact with the NR2D subunit, which is a key regulatory subunit of the NMDA receptor [53]. Collectively, these data suggest that regulation of NMDA receptor function may contribute to the neuroprotective effect of blocking the binding of oligomeric Aβ to PrP\(^C\). Furthermore, there is indirect evidence that PrP\(^C\) binding by oligomeric Aβ colocalizes with both mGlu5 (glutamate metabotropic subtype 5) and NMDA receptors [18]. Thus, the binding of PrP\(^C\)/Aβ may promote cross-linking of glutamate receptors. Interestingly, a recent study found that oligomeric Aβ increases the localization of PrP\(^C\) to the cell surface by increasing its trafficking [58]. Thus, oligomeric Aβ may induce the formation of ectopic signaling platforms by recruiting PrP\(^C\) at the plasma membrane [18]. Future studies are needed to clarify the detailed mechanisms by which PrP\(^C\) mediates Aβ-induced neurodegeneration. In addition, the effect of familial mutations in PrP\(^C\) and overexpression of PrP\(^C\) on Aβ-induced neurodegeneration remains to be determined.

In conclusion, I found that Prnp\(^{-/-}\) mice are more resistant to the neurotoxic effect of oligomeric Aβ than WT mice in both in vivo and in vitro models. Furthermore, the application of a specific anti-PrP\(^C\) antibody or competitive PrP\(^C\)
peptide, which block Aβ/PrP<sup>C</sup> binding, rescues Aβ oligomer induced neuronal cell death, demonstrating the requirement for PrP<sup>C</sup> in oligomeric Aβ-induced neurotoxicity. My results strongly support the concept that PrP<sup>C</sup> contributes to neurotoxic signaling induced by oligomeric Aβ, and mediates neuronal cell death.
Figure 2-5. Hypothetical model of Aβ oligomer-induced neurotoxic signaling through PrP<sup>C</sup>. Aβ oligomer binding to PrP<sup>C</sup> at plasma membrane activates NMDA receptors and subsequent caspase-3 dependent neuronal cell death. Treatment with anti-PrP<sup>C</sup> antibody or competitive PrP<sup>C</sup> peptides prevents the activation of NMDA receptor, suggesting that PrP<sup>C</sup>/Aβ oligomer interaction is a key mechanism of Aβ oligomer-induced neurotoxicity.
CHAPTER 3; Caveolin-1 Mediate Oligomeric Amyloid β-Induced Neurotoxic Signaling Through Cellular Prion Protein
Abstract

Numerous studies have shown that oligomeric amyloid-β (Aβ) plays a critical role in imitating Alzheimer’s disease although its pathogenic mechanism remains to be determined. Recently, the cellular prion protein (PrP^C) has been reported to be an essential co-factor in mediating the neurotoxic effect of oligomeric Aβ. However, the signaling mechanisms by which oligomeric Aβ induces neurotoxicity through PrP^C remain elusive. Using mouse hippocampal slice culture, I found that oligomeric Aβ induces NMDA receptor subunit 2B (NR2B) phosphorylation and Src-family activation in a manner dependent on PrP^C expression. Knockdown of caveolin-1 diminishes the interaction between PrP^C and Fyn, and inhibits oligomeric Aβ-induced NR2B phosphorylation. I then found that lack of caveolin-1 blocks oligomeric Aβ-induced neuronal cell death. Taken together, these data demonstrate that caveolin-1 mediate oligomeric Aβ-induced neurotoxic signaling through PrP^C.

Introduction

Alzheimer’s disease (AD) is the most prevalent form of dementia. Although the mechanisms remain unclear, considerable evidence in AD points to a causal role of oligomeric Aβ in the neurodegeneration. There is great interest in understanding mechanisms by which oligomeric Aβ affects neurotoxic signaling.
Recently, Lauren et al. demonstrated that the specific binding of oligomeric Aβ to cellular prion protein (PrP<sup>C</sup>) and that this interaction was essential for oligomeric Aβ-induced synaptic toxicity as determined by loss of long-term potentiation (LTP) [20]. Moreover, ablation of PrP<sup>C</sup> enhances cognitive function in transgenic mice overexpressing mutant APP genes linked to familial AD (APPswe and PS1ΔE9) and prevents early death and memory impairment [21]. A recent paper showed that oligomeric Aβ-induced signal is dependent on PrP<sup>C</sup>-expression in cells [54, 58]. Thus, while our recent study [45, 59], along with others, confirms the physical interaction between Aβ and PrP<sup>C</sup>, and PrP<sup>C</sup> mediates the neurotoxicity of oligomeric Aβ [46, 60], the exact signaling mechanisms by which PrP<sup>C</sup> mediate oligomeric Aβ-induced neurotoxicity remains elusive.

Fyn, a member of the Src family of tyrosine kinases (SFK) has been suggested to play roles in AD pathogenesis. Several groups showed that Aβ and Fyn may act synergistically <i>in vitro</i> and <i>in vivo</i> [61, 62], and that the distribution and levels of Fyn are altered in AD brains [63, 64]. In addition, reduction of Fyn in APP transgenic mice prevents Aβ toxicity, while overexpression enhances it [61, 65]. Fyn-tau complexes have been hypothesized to localize to postsynaptic densities to affect glutamate receptor function [66], and known to phosphorylate the NMDA receptor subunit 2B (NR2B) to facilitate interaction of the NMDAR complex with the postsynaptic density protein 95 (PSD-95) [65, 67], linking NRs to synaptic excitotoxic downstream signaling [67]. With respect to tyrosine kinase signaling, it is known that rises in intracellular calcium levels through NMDAR are
enhanced by tyrosine phosphorylation [68], involving neuronal cell toxicity and that inhibition of tyrosine kinases prevents induction of LTP [69].

In neurons, lipid rafts provide a platform for signal transduction initiated by several classes of neurotrophic factors [70]. In the region, there are a high concentration of receptors and other signaling molecules including Fyn kinase. Caveolin, which is a cholesterol binding protein and scaffolding protein found within membrane/lipid rafts [71], organizes certain neuronal growth-promoting proteins, such as neurotrophic receptor signaling pathways, to membrane/lipid rafts. Previous papers showed a caveolin-dependent coupling of PrP\(^C\) to the tyrosine kinase Fyn [72] and that this coupling was required for PrP\(^C\) stimulated signaling in bioaminergic cells [37]. Interestingly, caveolin protein levels in the hippocampus and caveolin mRNA in the frontal cortex is upregulated in AD [73]. I therefore hypothesized that caveolin-1 may contribute to oligomeric Aβ-induced neurotoxic signaling through PrP\(^C\).

Here, I report that caveolin-1 links the interaction, Fyn-PrP\(^C\) and is critical in oligomeric Aβ-induced neurotoxic signaling through PrP\(^C\). Together, these findings for the first time reveal the importance of caveolin-1 in oligomeric Aβ-induced neuronal cell toxicity and suggest that this pathway might play an important role in neurodegeneration in AD.

**Materials and Methods**

*Materials*
Anti-β-actin antibody was obtained from Millipore. Ifenprodil hemitartrate (NR2B antagonist) and PPDA (NR2D antagonist) were obtained from Ascent scientific. PP2, Src family kinases (SFK) inhibitor was purchased from Tocris bioscience. Anti-pERK1/2 polyclonal antibody, anti-ERK polyclonal antibody, anti-Fyn polyclonal antibody, p-SFK (Tyr416) polyclonal antibody, caveolin-1 antibody and p-NR2B (Tyr1472) antibody were obtained from Cell Signaling Technology. Protein A/G-agarose beads were obtained from Roche. Aβ peptide (1-42) and reverse transcription (42-1) were purchased from ANA spec. PrP (6D11) antibody was kindly gifted from Wen-Quan Zou (Case Western Reserve University, Ohio, USA). Propidium Iodide (PI) and other chemicals were obtained from Sigma Chemical Co.

Animals

Prnp<sup>−/−</sup> mice (ZürichI) [48] backcrossed onto the FVB/N background were obtained from George Carlson, McLaughlin Research Institute, Great Falls, Montana. Cav-1<sup>−/−</sup> mice were purchased from Jackson Laboratories (B6.Cg-Cav1tm1Mls/J).

Plasmid Constructs
A plasmid construct encoding caveolin-1 has been described in a previous paper [74]. Caveolin-1 and Fyn siRNA (siGENOMEsiRNA) were purchased from Dharmacon (Thermo).

**Preparation of Oligomeric Aβ**

Soluble oligomeric Aβ was prepared according to a previous paper [49]. Briefly, 1.0 mg of Aβ₁₋₄₂ peptide was dissolved in 120 µL of hexafluoroisopropanol for 60 min at room temperature, and placed back on ice for 5-10 min. Hexafluoroisopropanol was evaporated overnight in the hood at room temperature. The sample was dissolved by 100% DMSO by adding 20 µL fresh anhydrous DMSO (Sigma Hybri-Max) to 0.45 mg peptide, and diluted 5 mM peptide stock into medium. Diluted peptide was incubated at 4°C for 24 h, and then centrifuged at 14,000 g for 10 min in the cold. Before I treated slice culture with oligomeric Aβ, I incubated the oligomeric Aβ at room temperature for 20 h.

**Preparation of hippocampal slice cultures**

Organotypic hippocampal slice cultures were prepared according to a previous paper [50]. Briefly, hippocampal slice cultures were prepared from 7-10 day-old mouse pups. Slices were cut at 400 µm on a McIlwain tissue chopper, transferred to Millicell (Millipore Corp.) membrane inserts (0.4 µm), and placed in 6-well culture plated. The upper surfaces of the slices were exposed to a humidified 37°C
atmosphere containing 5% CO₂. Slice culture media consisted of basal Eagles medium with Earle’s balanced salt solution, 20% heat-inactivated horse serum, enriched with glucose to a concentration of 5.6 mM. The medium was changed every other day. Slices were examined periodically for viability, and any dark or abnormal slices were discarded.

**Experimental treatment of oligomeric Aβ to organotypic hippocampal slice culture**

The effects of oligomeric Aβ and other drugs were tested in the slices which had been maintained for 11-14 days in vitro. All reagents were added to serum free medium (no horse serum). Oligomeric Aβ was added to cultures in serum free medium. Vehicles were treated the same way except with reversed sequence of Aβ₁₋₄₂. At the indicated times after treatment initiation, slices were rinsed twice in cold phosphate buffered saline, and then harvested by removing the Millicell membrane insert.

**Assessment of neuronal cell death**

To determine neuronal cell death in the hippocampal slices, PI was added to the slice culture medium. Images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss). Nissl staining was also performed for routine histochemical and morphological analyses.
Protein extraction and Western blot analysis

The western blotting analysis was performed according to a previous paper [52]. After oligomeric Aβ treatment, these slices were rinsed twice with ice-cold PBS and then lysed in ice-cold lysis buffer. The protein concentration was determined by the method of BCA (Pierce). Equal amounts of sample proteins were separated according to their molecular weight on 10 or 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The blots were blocked with 10% milk in TBS-T for 1 h at room temperature, and then treated with primary antibodies diluted with 1% milk and incubated overnight at 4°C. The following antibodies were used: anti-p-ERK1/2 polyclonal antibody, anti-ERK polyclonal antibody, anti-Fyn polyclonal antibody, anti-p-SFK (Tyr416) polyclonal antibody, anti-caveolin-1 antibody, anti-p-NR2B (Tyr1472) antibody, β-actin antibody and PrP (6D11) antibody.

Co-immunoprecipitation

Homogenates were prepared from the brains of 2-3 month old mice in 50 mM Tris-HCl buffer (pH 7.5), containing 0.32 M sucrose, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM NaHCO₃ and NP-40. Samples containing 1 mg of protein were lysed for 30 min in 50 mM Tris-HCl buffer (pH 7.5), containing 150 mM NaCl, 0.5% NP-40, 1% β-octyl-D-glucopyranoside, 1 mM sodium fluoride, 2 mM NaVO₄, 0.1 mM PMSF, and EDTA-free protease inhibitor cocktail (Roche). The lysis buffer
containing this combination of detergents completely solubilizes lipid rafts and has been used in a number of studies involving coimmunoprecipitation of lipid raft components [75][76]. Samples were then centrifuged for 15 min at 14,000 rpm and 4°C. Supernatants were cleared with protein A/G-agarose beads (Roche) overnight at 4°C and incubated with pAbs against PrP, Fyn, caveolin-1 or non-immune rabbit IgG (for 1hr at 4°C), followed by precipitation with protein A/G-agarose beads overnight at 4°C. The beads were washed 6 times with RIPA buffer, once with PBS, and analyzed by immunoblotting.

Cell culture and transfections

Prnp<sup>+/+</sup>, Prnp<sup>−/−</sup> and GT1-7 cells were grown in OPTI-DME (Invitrogen) supplemented with 5% FBS and 1% penicillin/streptomycin (Invitrogen). Cells were transfected by Lipofectamine™ 2000 Transfection Reagent (Invitrogen) according to the manufacturer’s protocol. Cells were analyzed 72h after transfection. All cultures were kept at 37°C in a humidified 5% CO2-containing atmosphere.

Statistical analysis

Data were expressed as the means ± S.E.; the number of independent experiments is indicated in the corresponding figure legend. Differences between groups were examined for statistical significance using one-way analysis of
variance with an unpaired Students t-test. A $p$ value less than 0.05 indicated a statistically significant difference.

**Results**

*Oligomeric Aβ induce NR2B phosphorylation through Fyn in a PrP*$_C$* dependent manner in slice culture*

Src family kinases (SFK) and ERK play a key role in oligomeric Aβ-induced neuronal toxicity and neurons from mice deficient in Fyn were resistant to oligomeric Aβ-induced neurotoxicity [52, 62]. NR2B is a well known substrate of SFK, and its phosphorylation is suggested to mediate excitotoxicity [65, 77]. To confirm the phosphorylation of SFK, ERK and NR2B after oligomeric Aβ treatment, I used slice culture and analyzed the levels of p-SFK, p-ERK and p-NR2B. Oligomeric Aβ induces SFK activation 40 min after treatment, and ERK activation 6hr treatment (Figure 3-1a). NR2B phosphorylation appeared 40 min after oligomeric Aβ treatment and disappeared at once (Figure 3-1b). To clarify the relationship between SFK, ERK1/2 and NR2B phosphorylation, I pretreated slices with PP2, a selective SFK inhibitor, before the oligomeric Aβ treatment. As shown in Figure 3-1c and d, PP2 treatment prevents oligomeric Aβ-induced ERK activation and NR2B phosphorylation, suggesting that oligomeric Aβ induces NR2B phosphorylation through SFK. Among SFK, Fyn has been well-known to phosphorylate NR2B [65, 77]. Since there is no specific p-Fyn antibody available, I didn’t analyze phospho-Fyn level directly. To see the effect of Fyn kinase in
NR2B phosphorylation, I knockdown Fyn by RNAi in neuronal cell line and see the effect of oligomeric Aβ in NR2B phosphorylation in the absence of Fyn. As I had expected, knockdown of Fyn significantly reduced NR2B phosphorylation induced by oligomeric Aβ (Figure 3-1e), suggesting that Fyn, among SFK, plays a role in the NR2B phosphorylation.

Previous experiments have implicated PrP^C as a binding partner for oligomeric Aβ [20, 45, 78]. To test if oligomeric Aβ can initiate signal transduction events in a PrP^C-dependent fashion, I analyzed the expression level of p-SFK, p-ERK and p-NR2B after oligomeric Aβ treatment in slice culture from WT, Prnp^-/- animals. ERK1/2 and SFK phosphorylation is not altered upon treatment of slice culture from Prnp^-/- with oligomeric Aβ, whereas slices from wild type had an increase in ERK1/2 and SFK phosphorylation (Figure 3-1e). In addition, NR2B phosphorylation is only seen in WT but not Prnp^-/- (Figure 3-1f), suggesting that this signaling pathway and NR2B phosphorylation can be activated in a PrP^C-dependent way.
Figure 3-1. Oligomeric Aβ induces phosphorylation of NR2B through Fyn in a PrP<sup>C</sup>-dependent manner. a) Representative western blots showed oligomeric Aβ induces upregulation of p-SFK and p-ERK. Approximately equal loading of each lane was confirmed using actin antibody. b) Oligomeric Aβ induces a transient increase of p-NR2B in 40 min. c) and d) SFK inhibitor (PP2) pretreatment prevents oligomeric Aβ-induced ERK activation and NR2B phosphorylation. e) Fyn knockdown by RNAi significantly reduces phosphorylation of NR2B induced by oligomeric Aβ in PrP<sup>+/+</sup> cell line (*p<0.01). f) and g) Oligomeric Aβ induces an increase in p-SFK, p-ERK and p-NR2B levels in a dependent on PrP<sup>C</sup>. 
NR2B antagonist reduces oligomeric Aβ-induced neuronal cell death in slice culture

Previous findings show that oligomeric Aβ can activate extrasynaptic NR2B receptors and thereby contribute to the induction of long term depression (LTD) [79, 80]. I found that oligomeric Aβ induces NR2B phosphorylation through Fyn in a PrP<sup>C</sup> dependent manner (Figure 3-1e). I therefore proceeded to apply selective antagonists to test whether the neurotoxic effect of oligomeric Aβ on hippocampal slice culture was attributable to a specific NMDA receptor subtype. A specific NMDA antagonist, (2R)-amino-5-phosphonopentanoate (APV), blocked the oligomeric Aβ-induced neurotoxicity (Figure 3-2). Interestingly, the selective NR2B antagonist ifenprodil (3 μM) also reduced oligomeric Aβ-induced neurotoxicity, while NR2D antagonist PPDA (1 μM) didn’t rescue the neuronal cell death in slice culture (Figure 3-2). This data suggest that the activation of NR2B-containing receptors may contribute to oligomeric Aβ-induced neuronal cell death in slice culture.
Figure 3-2. Treatment with an NR2B antagonist prevents oligomeric Aβ-induced neuronal cell death. Hippocampal slices were incubated with NMDA antagonist, APV (50 µM), selective NR2B antagonist ifenprodil (3 µM) or NR2D antagonist PPDA (1 µM) for 1 h and then treated with oligomeric Aβ (500 nM) in the presence of PI for 48 h. PI staining was analyzed with microscopy to determine the effects of these pharmacological agents on oligomeric Aβ-induced toxicity. APV and ifenprodil significantly inhibited oligomeric Aβ-induced neuronal cell death (n=5). Scale bar, 500 µm. (**p<0.01 versus control, #p<0.05 versus Aβ treatment only).
Fyn kinase and caveolin-1 interact with PrP<sub>C</sub> in vivo

Fyn kinase during signal transduction events is noncovalently associated with glycosylphosphatidylinositol (GPI)-anchored proteins [81]. Co-immunoprecipitation experiments using whole mouse brain lysates and antibodies to PrP<sub>C</sub> and Fyn provided evidence for the interaction of these proteins (Figure 3-3a and b). Because Fyn is an intracellular protein and PrP<sub>C</sub> is located at outer plasma membrane, these two proteins interaction are likely to involve intermediate factor(s). Previous papers suggested that caveolin-1 takes part in PrP<sub>C</sub>-mediated Fyn activation in neuronal differentiation model [37, 72]. In addition, a previous study reported that caveolin-1 interacts with PrP<sub>C</sub> [82]. Furthermore, in integrin signaling, a Ras-ERK pathway has been shown to depend on the recruitment of the caveolin-Fyn complex [83]. Therefore, I reasoned that caveolin-1 links the interaction between Fyn and PrP<sub>C</sub>. Co-immunoprecipitation experiments show that Fyn interacts with caveolin-1 (Figure 3-3c) and caveolin-1 interacts with PrP<sub>C</sub> in mouse brain homogenate (Figure 3-3d).

Caveolin-1 mediate the interaction between Fyn and PrP<sub>C</sub>

To further investigate the role of caveolin-1 in linking PrP<sub>C</sub> and Fyn, I analyzed the interaction in two cell lines, which have either caveolin-1 or no caveolin-1 expression. Neuronal Prnp<sup>+/+</sup> cell line [84] expressed caveolin-1, while, consistent with a previous report [72], GT1-7 has no caveolin-1 expression with a similar PrP expression to Prnp<sup>+/+</sup> cell line (Figure 3-4a). Co-
immunoprecipitation with Fyn shows that Fyn failed to bind with PrP in GT1-7 cell line which has no caveolin-1 expression, whereas there is a clear interaction in Prnp\(^{+/+}\) cell line (Figure 3-4b). I confirmed that immunoprecipitated Fyn levels were equal in each cell line (Figure 3-4b).

I re-construct caveolin-1 in GT1-7 (Figure 3-4c) and analyzed Fyn-PrP\(^C\) interaction by co-immunoprecipitation. Co-immunoprecipitation data shows that caveolin-1 re-construction in GT1-7 cell established Fyn-PrP\(^C\) interaction (Figure 3-4d), while control transfection didn’t induce the interaction. To further confirm that caveolin-1 links these two proteins interaction, I knockdown caveolin-1 in Prnp\(^{+/+}\) cell line with siRNA and performed co-immunoprecipitation experiment. Western blots data shows that caveolin-1 RNAi reduces caveolin-1 expression by 85\% in Prnp\(^{+/+}\) cell line (Figure 3-4e). Co-immunoprecipitation with Fyn data shows that PrP\(^C\)-Fyn interaction is impaired by caveolin-1 knockdown (Figure 3-4f). These data here shows that caveolin-1 acts as a scaffold protein for PrP\(^C\) and Fyn.
Figure 3-3. Interaction of PrP<sup>C</sup> with caveolin-1 and Fyn. a) Immunoblot analysis detected PrP<sup>C</sup> in Fyn and PrP<sup>C</sup> immunoprecipitates (IP) of mouse whole brain homogenate. b) Immunoblot with Fyn in PrP<sup>C</sup> IP shows the interaction of PrP<sup>C</sup> and Fyn in mouse brain homogenate. c) Immunoblot analysis detected caveolin-1 in Fyn IP of mouse whole brain homogenate from Prnp<sup>+/+</sup> and Prnp<sup>−/−</sup> mouse. d) Immunoblot with PrP in caveolin-1 IP shows the interaction of PrP<sup>C</sup> and caveolin-1 in WT brain homogenate. No precipitation was observed from Prnp<sup>−/−</sup> mouse brain homogenate.
Figure 3-4. Caveolin-1 mediates PrP$^C$ and Fyn interaction. a) GT1-7 cell line has no caveolin-1 expression, while PrP$^C$ expression level in GT1-7 is similar to that in Prnp$^{+/+}$ cell line. Approximately equal loading of each lane was confirmed using actin antibody. b) Immunoblot with PrP$^C$ in Fyn IP of GT1-7 cells or Prnp$^{+/+}$ cell line shows that GT1-7 cells have no PrP$^C$-Fyn interaction. The equal amount of immunoprecipitated Fyn was confirmed by the immunobLOTS with Fyn. c) Caveolin-1 cDNA was transfected in GT1-7 cells. 48 hours post-transfection, cell lysates were prepared and subjected to SDS-PAGE/Western blot analysis with anti-caveolin-1. The transfection increase the expression of caveolin-1 compared to mock plasmid transfection in GT1-7 cell line. d) Co-immunoprecipitation with Fyn shows that caveolin-1 transfection induces Fyn and PrP$^C$ interaction in GT1-7 cell line. e) Caveolin-1 siRNA was transfected in Prnp$^{+/+}$ cell line. 72 hrs after transfection, cell lysates were subjected to western blot analysis with anti-caveolin-1. Caveolin-1 expression was reduced by approximately 85%. Approximately equal loading of each lane was confirmed using actin antibody. f) Co-immunoprecipitation with Fyn shows that the interaction of PrP$^C$-Fyn was reduced in Prnp$^{+/+}$ cell line transfected with caveolin-1 siRNA.
Caveolin-1 is required for oligomeric Aβ-induced NR2B phosphorylation

I found that caveolin-1 plays a role as physically linking Fyn and PrP^C proteins. Therefore, I reasoned that the interaction may influence oligomeric Aβ-induced signaling. To analyze whether caveolin-1 plays a role in the phosphorylation of Y1472 NR2B, I introduce caveolin-1 RNAi to Prnp^{+/+} cell line and analyzed the responses after oligomeric Aβ challenge. Importantly, knockdown of caveolin-1 reduced oligomeric Aβ-induced NR2B phosphorylation (Figure 3-5a). To further investigate the role of caveolin-1 in the NR2B phosphorylation, I reconstructed caveolin-1 in GT1-7 cells and analyzed NR2B phosphorylation after oligomeric Aβ treatment. While oligomeric Aβ didn’t increase NR2B phosphorylation in GT1-7 cell line, oligomeric Aβ induced NR2B phosphorylation after transfection (Figure 3-5b). Thus, caveolin-1 appears to be one of the protagonists involved in oligomeric β-induced NR2B phosphorylation.
Figure 3-5. Knockdown of caveolin-1 reduces oligomeric Aβ-induced NR2B phosphorylation. a) Prnp\(^{+/+}\) cell line was transfected with caveolin-1 siRNA and treated with oligomeric Aβ (500 nM) for an indicated time points. Oligomeric Aβ-induced p-NR2B was only seen in control transfection (*p<0.05, n=3). b) GT1-7 cells were transfected with caveolin-1 cDNA and treated with oligomeric Aβ (500 nM) for an indicated time points. Caveolin-1 expression was confirmed by western blots with anti-caveolin-1. Approximately equal loading of each lane was confirmed using actin antibody. Significant increase of p-NR2B by oligomeric Aβ was seen in GT1-7 cell line with caveolin-1 cDNA transfection (*p<0.05, n=3).
Lack of caveolin-1 is resistant to oligomeric Aβ-induced neurotoxicity in slice culture

I at last examined the effect of oligomeric Aβ on neuronal cell death in hippocampal slice cultures prepared from wild type (WT) or Cav-1−/− mice (Figures 3-6a) to test whether caveolin-1 mediates oligomeric Aβ-mediated neuronal cell death. To confirm my finding in vitro that caveolin-1 links the interaction between PrP and Fyn, I performed co-immunoprecipitation in the brain homogenate from WT or Cav-1−/− mice with anti-Fyn or anti-PrP. Co-immunoprecipitation data shows that the interaction was dramatically diminished in Cav-1−/− mice (Figure 3-6b), suggesting that caveolin-1 mediates the interaction between Fyn and PrP in vivo, which is consistent with cell culture data. Next, I treated slice culture from WT or Cav-1−/− mice with oligomeric Aβ and analyzed p-SFK and p-NR2B. Oligomeric Aβ induces both SFK and NR2B phosphorylation at 40 min after treatment in WT slice culture, while no phosphorylation was seen in Cav-1−/− slice culture. To further investigate the role of caveolin-1 in oligomeric Aβ-induced neurotoxicity, I analyzed the neuronal cell death in slice culture. Consistent with previous studies [59], oligomeric Aβ (500 nM) induced significant neuronal cell death in WT samples, as measured by PI staining (Figure 3-6d). However, the neuronal cell death induced by oligomeric Aβ was significantly decreased in slice cultures prepared from Cav-1−/− mice. In addition, activation of caspase-3 after oligomeric Aβ treatment was attenuated in
slices from Cav-1<sup>−/−</sup> mice (Figure 3-6e). Thus, caveolin-1 mediates oligomeric Aβ-induced neurotoxic signaling and is required for its neurotoxicity.
Figure 3-6. Caveolin-1 deficiency prevents oligomeric Aβ-induced neuronal cell death. a) Total protein (20 μg) from whole brain of the indicated genotype was analyzed by immunoblot with anti-caveolin-1. Immunoblot analysis shows caveolin-1 band in WT samples, and no band in Cav-1−/− samples. Approximately equal loading of each lane was confirmed using actin antibody. b) Co-immunoprecipitation with anti-Fyn and anti-PrPC shows that Fyn-PrPC interaction is disrupted in Cav-1−/− animals. c) Oligomeric Aβ induces p-SFK and p-NR2B in a dependent on caveolin-1 in slice culture. d) Representative pictures showed PI staining induced by oligomeric Aβ was significantly reduced in slice cultures from Cav-1−/− mice compared to Cav-1+/+ mice. The PI staining were quantitatively analyzed (n=5). Scale bar, 500 μm (**p<0.01). e) Representative western blot data shows that the expression of active caspase-3 is significantly reduced in Cav−/− slices 24 h after oligomeric Aβ treatment (n=4). (**p<0.01, *p<0.05).
Discussion

In this study, I clarify the signaling mechanisms by which oligomeric Aβ induces neurotoxicity through PrP^C. My data demonstrate that caveolin-1 mediates the PrP^C-Fyn interaction and that loss of caveolin-1 attenuates oligomeric Aβ-induced NR2B phosphorylation by Fyn, followed by neuronal cell death. Collectively, caveolin-1 acts as a scaffold protein for Fyn and PrP^C, and is critical in oligomeric Aβ-induced neurotoxic signaling (Summarized in Figure 3-7). To my knowledge, my findings provide the first evidence for the critical role of caveolin-1 in oligomeric Aβ-induced neurotoxic signaling.

Accumulated evidences suggested that oligomeric Aβ appears to disturb synaptic plasticity in neurons and affect memory in a mouse model of AD in a PrP^C dependent manner [20, 21, 46]. However, three independent studies failed to confirm a critical role of PrP^C in vivo and in vitro [22-24]. This conflict may come from the difference of experimental conditions, such as animal models or oligomeric Aβ preparation. In spite of this, it is well established that oligomeric Aβ binds to PrP^C. My very recent study strongly supports a critical role for PrP^C in mediating the neurotoxic effect of oligomeric Aβ and that the PrP^C/Aβ interaction is necessary for triggering neuronal cell loss in vitro and in vivo [59]. Furthermore, a recent study indicates that PrP^C can mediate toxic signaling of various β-sheet-rich conformers, such as oligomeric Aβ, independent of infectious prion propagation and that the neurotoxicity was significantly blocked by NMDA antagonist treatment [54], indicating the involvement of NMDA receptor in the
neurotoxicity. In this study, I therefore confirmed that neurotoxic signaling induced by oligomeric Aβ is dependent on PrP<sup>C</sup> expression. Oligomeric Aβ-induced phosphorylation of Fyn, ERK and NR2B were dramatically attenuated in Prnp<sup>−/−</sup> slice culture, suggesting that PrP<sup>C</sup> plays an important role in oligomeric Aβ-induced neurotoxic signaling.

NMDA receptors have been considered to be involved in excitotoxicity mediated neuronal loss in several neurodegenerative conditions [77]. Previous papers suggested that NMDA receptor-mediated excitotoxicity might be a downstream mechanism of Aβ neurotoxicity [17, 54], which is further supported by my findings that nonspecific NMDA antagonist or NR2B specific antagonist blocked oligomeric Aβ-induced neuronal toxicity. In addition, oligomeric Aβ induces NR2B phosphorylation (Tyr1472) by Fyn in a dependent on PrP<sup>C</sup> expression. These data suggest that the abnormal upregulation of NR2B function may contribute to the oligomeric Aβ-induced neurotoxic effect through PrP<sup>C</sup>. Several studies show that NR2A is generally found at the synapse, whereas NR2B is predominantly localized at extrasynaptic sites [85] and enhanced activation of extrasynaptic NR2B-containing NMDARs is common in AD. The level of tyrosine phosphorylation of NR2B is tightly linked to receptor activity and neuronal cell death. Enhanced activation of extrasynaptic NR2B leads to excessive influx of Ca<sup>2+</sup> into the cell, which results in inappropriate activation of enzymes (such as calpains and other calcium-regulated enzymes) and mitochondrial dysfunction; this leads to apoptosis [68, 85]. Phosphorylation of NR2B is likely to suppress NMDA receptor internalization and increase the
NR2B levels at plasma membrane [86, 87], leading to the enhancement of signaling and Ca\(^{2+}\) influx through NR2B. Furthermore, inhibition of NR2B phosphorylation protects glutamate-induced neurotoxicity [88] and long term potentiation (LTP) [69]. Thus, the increase in NR2B Tyr1472 phosphorylation and the subsequent enhancement of NMDA receptor-mediated calcium entry following oligomeric A\(\beta\) treatment may be a part of the molecular mechanisms underlying neurotoxicity induced by oligomeric A\(\beta\). Interestingly, A\(\beta\) increases glutamate release from astrocytes and microglia [89][90], which may play a synergic role in excitotoxicity through NR2B.

PrP\(^C\) is a GPI-anchored protein, and as such it may need to interact and modulate oligomeric A\(\beta\) to transduce extracellular signals leading to Fyn activation. I found that caveolin-1, acting as a scaffolding protein, plays a critical role in oligomeric A\(\beta\)-induced neuronal toxic signaling. Caveolin-1 is expressed in multiple types of neurons, including hippocampal and dorsal root ganglion neurons, as well as in excitatory synapses in the CA1 of neonatal and adult hippocampus, all of which express glutamate receptors [36, 91]. My data shows that oligomeric A\(\beta\)-induced Fyn activation, NR2B phosphorylation and neurotoxicity are dependent on caveolin-1 expression, suggesting that caveolin-1 is necessary for the neurotoxic signaling. To support my idea, previous papers showed that the C-terminal half of the oligomerization domain (i.e. residues 82–101 in Cav-1) binds to and regulates the activity of signaling molecules, including receptor tyrosine kinases and their downstream targets (e.g. EGFR, c-Neu, Ha-Ras, MEK, and extracellular signal-regulated kinase) [92, 93]; non-receptor
tyrosine kinases (e.g. Src and Fyn) [83]. In addition, caveolin-1 is required for PrP<sup>C</sup>-induced Fyn activation [72] and that this coupling was required for PrP<sup>C</sup> stimulated signaling in bioaminergic cells [37]. A general scaffolding role of caveolin-1 is well accepted, although some opposite effects have been observed. In particular, neuron-targeted caveolin-1 protein enhances prosurvival and pro-growth signaling [94]. However, I did not observe any difference of signaling pathways in basal levels or after NMDA treatment in Cav<sup>-1/-</sup> animals compared to Cav<sup>++</sup> (Data not shown), suggesting that NMDA-R sensitivity is not altered by knockout of caveolin-1 in my model. This discrepancy may come from the difference of experimental models.

My data also suggests the importance of lipid rafts in oligomeric Aβ-induced neurotoxic signaling. Interestingly, Aβ is highly concentrated in lipid rafts, which comprise a small fraction of brain volume but contain 27% of brain Aβ<sub>1-42</sub> and 24% of Aβ<sub>1-40</sub> in young mice [95] and the accumulation might be a key in Aβ-induced cell death [96]. Ehehalt et al. reported that lipid rafts are critically involved in regulating the secretase cleavage that produces Aβ [97]. Interestingly, while enhanced cholesterol levels increase caveolin-1 expression [98] and exacerbates Aβ-induced neurotoxicity [99], cholesterol depletion inhibits the generation of Aβ [100] and protects cells from Aβ-induced neurotoxicity [101]. One of the most remarkable findings of the current study is my observation that caveolin-1 is a critical protein for oligomeric Aβ-induced neurotoxicity through PrP<sup>C</sup> in hippocampus slice culture. My results strongly support the concept that PrP<sup>C</sup> mediates oligomeric Aβ-induced neurotoxic signaling and indicates that
oligomeric Aβ can induce cross-link PrP\textsuperscript{C}, leading to activation of Fyn and NR2B, and neuronal cell death. The modification of signaling pathways mediated by PrP\textsuperscript{C} may provide a novel therapeutic approach for AD.
Figure 3-7. Schematic model of neurotoxic signaling induced by oligomeric Aβ through PrP<sup>C</sup>. Aβ binds to PrP<sup>C</sup> at plasma membrane and induces the activation of Fyn through caveolin-1. Activated Fyn phosphorylates NR2B, following by Ca<sup>2+</sup> influx. The enhanced Ca<sup>2+</sup> influx results in inappropriate activation of enzymes (such as calpains and other calcium-regulated enzymes), leads to apoptotic pathways.
CHAPTER 4: Toxicity of Soluble Amyloid Oligomers through Neuronal Cell Cycle Re-entry
Abstract

Accumulating evidence suggests that aberrant neuronal cell cycle re-entry precede the selective neurodegeneration observed in Alzheimer disease (AD). While the causal role of cell cycle alteration in the pathogenesis of AD remains to be determined, our recent animal model study clearly demonstrated that dysregulation of cell cycle re-entry results in neurodegeneration in vivo suggesting the causal link between cell cycle re-entry and neuronal cell loss in AD. Therefore, the re-activation of cell cycle in the vulnerable neurons in AD might be an essential part of mechanism leading to neuronal cell death. However, the signaling mechanism(s) associated with cell cycle re-entry and neuronal cell death in AD is unclear and needs to be identified. In this study, I investigated the intracellular signaling mechanisms triggered by the oligomeric Aβ and determined the causal relationship among these pathways with the oligomeric Aβ-mediated neurotoxicity in the organotypic hippocampal slice cultures. I found that oligomeric Aβ causes cell cycle re-entry in hippocampal slice cultures and subsequent neuronal cell death. Initially, oligomeric Aβ increases intracellular Ca²⁺ through NMDA receptor and this event activates calcium/calcium-dependent protein kinase (CaMKII), which subsequently activates the extracellular signal-regulated protein kinase (ERK1/2) signaling pathways. The activated ERK1/2 induces p27Kip degradation, which leads to cell cycle re-activation. Importantly, inhibition of ERK/CaMKII signaling pathway and cell cycle re-entry significantly attenuate neuronal cell death induced by oligomeric
Aβ. Furthermore, I found that deficiency of E2F1, ATM or p53 is more resistant to oligomeric Aβ-induced cell death than their wild-type littermates.

Taken together, these results demonstrate the central role of cell cycle re-entry in oligomeric Aβ mediated neuronal cell death. I also showed that the CaMKII-ERK1/2 signaling pathway, by mediating the degradation of p27Kip, is a key mechanism for neuronal cell cycle re-entry. Furthermore, DNA damage responses through ATM kinase-p53 may contribute to cell death through cell cycle re-entry. Inhibition of cell cycle re-entry signals may therefore provide strategies for neuroprotection in AD.

**Introduction**

Accumulating evidence suggests that aberrant neuronal cell cycle re-entry may precede the regional neurodegeneration observed in AD [38-40]. In mature neurons, the cell cycle is normally arrested at the G0 phase, but the lines of evidence indicate the upregulation of various cell cycle markers in neurons vulnerable to degeneration in AD while no evidence exists of actual mitosis [41]. This suggests that these neurons are arrested at a point(s) prior to the physical event of cellular division, G2-M phase, and once cyclin A is expressed, the arrested cells lack the ability to return to G0 and therefore must either complete the cycle or die [102]. Although the causal relationship between this cell cycle alteration and neurodegeneration in AD is unclear, our recent study clearly demonstrated that dysregulation of cell cycle re-entry results in neurodegeneration.
in vivo [42], suggesting the re-activation of cell cycle in the vulnerable neurons in AD might be an essential part of mechanism leading to neuronal cell death. However, the exact signaling mechanisms associated with cell cycle re-entry and neuronal cell death in AD remains to be elucidated. Importantly, it has been shown that oligomeric Aβ, but not Aβ monomers, can directly induce neuronal cell cycle induction in primary cortical neurons [43]. However, few studies have tried to understand how the oligomeric Aβ affect neuronal cell cycle and how the oligomeric Aβ may modulate this process at the molecular level. Furthermore, there is little known about the precise role and molecular basis of the oligomeric Aβ in the modulation of neuronal loss.

Considering the effect of the oligomeric Aβ on cell cycle re-entry and neuronal cell death, several pathways might be involved in both mechanisms such as the mitogen-activated protein kinase (MAPK), phosphatidylinositol 3’-kinase/Akt, protein kinase C, and Ca++/calmodulin-dependent protein kinase (CaMKII) pathways [103]. These pathways are known to influence the expression of cell cycle regulators, such as cyclins and cyclin-dependent kinases, and thus initiate G1/S phase transition and cell cycle re-entry [104]. Specifically, previous observation suggests a potential link between CaMKII and a cell cycle regulation. A synthesized CaMKII inhibitor, for example, causes cell cycle arrest at the G1 phase in NIH 3T3 cells [105], and, importantly, a recent study showed that CaMKII promotes cell cycle progression through activation of extracellular signal-regulated protein kinase (ERK1/2) and subsequent modulation of p27Kip phosphorylation in human colon adenocarcinoma cells [106]. p27Kip is a cell cycle
inhibitor that regulates cell cycle transitions by binding to and regulating the activity of cell cycle dependent kinases. In fact, the increased level of phosphorylated p27Kip and the active form of ERK1/2 are found in the vulnerable neurons in AD [38, 107]. More interestingly, the ERK1/2 cascade was known to be activated in vitro model for Aβ toxicity [52].

DNA replication stress has been known to induce DNA damage in several cell lines [108]. Previous papers suggested that activation of DNA replication may contribute to cell cycle-induced cell death in neurons though DNA damage [109], and neurons have been shown to be quite vulnerable to DNA damage condition [110]. Importantly, in AD brains, our group found an upregulation of gamma H2AX, an important indicator of DNA damage preceding apoptotic changes, in the hippocampal regions [111].

Therefore, I hypothesized that oligomeric Aβ may induce cell cycle re-entry through CaMKII-ERK1/2 signaling and, ultimately, cause neuronal cell death in hippocampus via DNA damage response. To test this hypothesis, in this study, I investigated the intracellular signaling mechanisms triggered by the oligomeric Aβ and determined the causal relationship among these pathways with the oligomeric Aβ-mediated neurotoxicity in the organotypic hippocampal slice cultures and in vivo model. Furthermore, I investigated molecular mechanisms by which cell cycle re-entry induces neuronal cell death. Thus, my data suggest that G1 transit and S-phase entry through CaMKII-ERK1/2 signaling are critical factors in oligomeric Aβ-induced neuronal apoptosis, and the ATM-p53 response
may function to induce apoptotic pathway during inappropriate neuronal cell cycle progression.

Materials and Methods

Materials

A specific protein kinase inhibitor, U0126 that inhibits MEK1/2 activation of ERK was obtained from Biomol Research. Anti-β-actin antibody and anti-NeuN mouse monoclonal antibody were obtained from Millipore. Anti-proliferating cell nuclear antigen (PCNA) rabbit polyclonal antibody, anti-MAP2 antibody and anti-cyclin A rabbit polyclonal antibody were obtained from Abcam. Anti-caspase 3 rabbit polyclonal antibody and anti-phospho-p27Kip are purchased from Santa Cruz Biotechnology. Anti-p27Kip mouse monoclonal antibody was obtained from Invitrogen. Anti-pERK1/2 polyclonal antibody, anti-ERK polyclonal antibody and anti-cdk4 monoclonal antibody were obtained from Cell Signaling Technology. Aβ peptide (1-42) was purchased from California Peptide Research, Inc. Propidium Iodide (PI), KN-93 (a specific CaMKII inhibitor), Flavopiridol hydrochloride (cyclin-dependent kinase inhibitor) and other chemicals were obtained from Sigma Chemical Co.

Preparation of oligomeric Aβ
Soluble oligomeric Aβ were prepared as described previously [49]. Briefly, 1.0 mg of Aβ1-42 peptide was dissolved in 120 µL of hexafluoroisopropanol for 60 min at room temperature, and placed back on ice for 5-10 min. Hexafluoroisopropanol was evaporated overnight in the hood at room temperature. The sample was dissolved by 100% DMSO by adding 20 µL fresh anhydrous DMSO (Sigma Hybri-Max) to 0.45 mg peptide, and diluted 5 mM peptide stock into medium. Diluted peptide was incubated at 4°C for 24 h, and then centrifuged at 14,000 g for 10 min in the cold. Before I treated slice culture with oligomeric Aβ, I incubated the oligomers at room temperature for 20 h.

Preparation of hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as described previously [50]. Briefly, hippocampal slice cultures were prepared from 7-10 day-old mouse pups. Slices were cut at 400 µm on a McIlwain tissue chopper, transferred to Millicell (Millipore Corp.) membrane inserts (0.4 µm), and placed in 6-well culture plated. The upper surfaces of the slices were exposed to a humidified 37°C atmosphere containing 5% CO2. Slice culture media consisted of basal Eagles medium with Earle’s balanced salt solution, 20% heat-inactivated horse serum, enriched with glucose to a concentration of 5.6 mM. The medium was changed every other day. Slices were examined periodically for viability, and any dark or abnormal slices were discarded.
Experimental treatment of oligomeric Aβ to organotypic hippocampal slice culture

The effects of oligomeric Aβ and other drugs were tested in the slices which had been maintained for 11-14 days in vitro. All reagents were added to serum free medium (no horse serum). Oligomeric Aβ was added to cultures in serum free medium. Vehicles were treated the same way except with reversed sequence of Aβ1-42. At the indicated times after treatment initiation, slices were rinsed twice in cold phosphate buffered saline, and then harvested by removing the Millicell membrane insert.

Assessment of neuronal cell death by PI staining

To analyze the degree of hippocampal neuronal cell death, hippocampal slices were stained by adding PI into slice culture medium at a concentration of 5 µg/ml. At indicated times after oligomeric Aβ treatment, the degree of hippocampal neuronal death were evaluated by microscopic observation of PI staining as described previously [52]. Images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss). The intensity of the fluorescence was quantitatively analyzed using Scion Image. The images were expressed as an arbitrary unit of PI staining.

Lactate Dehydrogenase Activity
LDH enzymatic activity in the culture medium was used to evaluate the extent of cellular damage produced in cultured slices subjected to the different treatments as described previously [52]. Briefly, culture medium was collected after incubation of slices, as indicated in the figures, and from the vehicle-treated control cultures. 70 μl aliquots of culture media were taken for determination of LDH activities using LDH assay kit (Roche) according to the manufacturer’s directions. The activity was expressed as the relative percentage of neuronal death using respective values for vehicle-treated slice cultures as 100%.

*Terminal dUTP Nick-End Labeling (TUNEL) Analysis*

Detection of 3’-OH termini of DNA strand breaks was performed on slice culture using an in situ cell death detection kit (Roche) following the recommendations of the manufacturer. Briefly, the tissue sections were treated with proteinase K (20 μg/ml in 10 mmol/L Tris-HCl, pH 7.4) for 30 minutes at 37°C after rehydration. After rinsing slides with PBS, TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescence-labeled nucleotide was applied for 1 hour at 37°C. After the incubation, sections were washed and mounted using aquamount (Southern Biotech). Positive signals were observed using fluorescence microscopy.

*Protein extraction and Western blot analysis*
The western blotting analysis was performed as described previously [52]. After oligomeric Aβ treatment, these slices were rinsed twice with ice-cold PBS and then lysed in ice-cold lysis buffer. The protein concentration was determined by the method of BCA (Pierce). Equal amounts of sample proteins were separated according to their molecular weight on 10 or 12% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The blots were blocked with 10% milk in TBS-T for 1 h at room temperature, and then treated with primary antibodies diluted with 1% milk and incubated overnight at 4°C. The following antibodies were used: anti-phosphorylated ERK1/2 (1:1000; Cell Signaling), anti-ERK1/2 (1:1000; Cell Signaling), anti-cdk4 (1:1000; Cell signaling), anti-p27Kip (2 µL/mL; Invitrogen), anti-phosphorylated-p27Kip (1:200; Santa Cruz), anti-PCNA (1:1000; Santa Cruz), anti-cyclin A (1:1000; Abcam), anti-activated CaMKII (1:1000; Promega), anti-caspase-3 (1:1000; Santa Cruz).

Immunocytochemistry

Cultured slices were rinsed with cold phosphate buffer saline (PBS) once and fixed for 2 h with 4% paraformaldehyde in 0.1 M phosphate buffer. After washing with PBS, sections were permeabilized overnight with PBS containing 0.2% Triton X-100. At the end of the permeabilization blocking solution (10% normal goat serum in PBS) was added to the slices for 4 h at room temperature. After washing with PBS, primary antibody was added and incubated for 24-48 h at 44°C. The following antibodies were used: anti-pBS, secondary antibody (either
Alexa 488 or 568) at a dilution of 1:300 was added and incubated for 4 h at room temperature. Counterstaining was then performed with 4’, 6-diamidino-2-phenylindole (DAPI). After the incubation, sections were washed and mounted using aquamount (Southern Biotech). Nissl stains were also performed for routine histochemical and morphological analyses.

*BrdU incorporation analysis*

5rdU incorporation analysis (normal goat serum in PBS) was added to the slices at µM in the presence and absence of oligomeric Aβ. For the visualization of the BrdU marker, slices were fixed at 24 h post treatment in 4% paraformaldehyde at 4°C. The following antibodies were used: anti-d in saline before permeabilization for overnight at 4°C in 0.4% Triton-X. Primary antibodies (BrdU antibody; 1:1000) were applied overnight at 4°C in saline. Following extensive washings, donkey Alexa-568 secondary antibodies (Molecular Probes) were then applied for 4 h at a concentration of 1:300.

*Oligomeric Aβ intrahippocampal injection*

Adult mice (2 months old) were anaesthetized with pentobarbital and placed in a stereotaxic frame. Injection was made through a 10 μl microsyringe (Hamilton). A volume of 1 μl Oligomeric Aβ dissolved at 50 μM was injected
into the left hippocampus. Control animals were prepared identically and injected with the same volume of \( \text{A}\beta_{42-1} \) (reversed sequence of \( \text{A}\beta_{1-42} \)). Injections were made at stereotaxic coordinates of Bregma; anteroposterior (AP)= 2.3mm, mediolateral (ML)=2.5mm, dorsoventral (DV)=-2.5mm. This corresponds to a site in the dorsal hippocampus in the apical dendritic zones of the CA1 region near the hippocampal fissure. Animals typically recovered from anesthesia after 2 h. Those animals were sacrificed 3, 5, 10, 20 days after injection.

**Statistical analysis**

Data were expressed as the means ± S.E. of the values from the number of experiments indicated in the corresponding figures. Differences between groups were examined for statistical significance using one-way analysis of variance with an unpaired Students t-test. A \( p \) value less than 0.05 denoted the presence of a statistically significant difference.

**Results**

*Treatment with Oligomeric A\( \beta \) Causes Cell Death in Organotypic Hippocampal Slice Cultures*

To examine oligomeric A\( \beta \) neurotoxicity in the hippocampal slice cultures, I analyzed the level of cell death by measuring PI staining. PI staining has been...
used as a reliable quantitative method of neuronal cell death in the hippocampal slice cultures [112]. As shown in Figure 4-1a, the oligomeric Aβ caused a time-dependent increase in PI staining in the hippocampal CA regions. The PI staining was first detectable after 24 h treatment of oligomeric Aβ and continued to increase at 48 h, indicating the progressive neuronal cell death. However, there was no cell death in slices culture without oligomeric Aβ treatment. I also observed that these cell death were seen in an oligomeric Aβ concentration dependent manner (data not shown). As shown in Fig. 4-1b, in consistent with PI staining data, the amount of LDH in the media containing hippocampal slices treated with the oligomeric Aβ was significantly increased in a time-dependent manner above control levels. These data are consistent with previous observation in a rat slice culture model [52]. Indeed, Nissl stain confirmed massive and selective neuronal loss in the CA3 and DG region in oligomeric Aβ-treated slices, but not in control slices (Figure 4-1c). To expand on these findings, I assessed TUNEL staining in the hippocampal slices after oligomeric Aβ treatment to measure apoptotic changes. Consistent with the PI staining data, increased numbers of TUNEL-positive neurons were found in the hippocampus with oligomeric Aβ treatment for 48 h (Figure 4-1d) and those positive cells are completely overlapped with PI positive cells. Caspase-3 activation was seen in slices after oligomeric Aβ treatment (Figure 4-1e). Furthermore, I previously showed that caspase inhibitor blocks the neuronal cell death (Figure 2-3). These data indicates that oligomeric Aβ induces neuronal cell death via apoptosis pathway.
Figure 4-1. PI staining and caspase-3 activation upon exposure to oligomeric Aβ. a) Time dependent response of PI staining in hippocampal slices were recorded over 48 h incubation after exposing hippocampal slice cultures to 5 µM oligomeric Aβ for the indicated times. The PI staining responses were quantitatively analyzed and shown as graphs. Scale bar, 500 µm. b) In time course experiments over a 48-h incubation, LDH activity in cultured media from hippocampal slices were assayed after exposing hippocampal slice cultures to oligomeric Aβ for the indicated times. c) Nissl staining clearly demonstrates the selective neurodegeneration in the CA3 region in oligomeric Aβ treated slices, but not in control slices. Scale bar, 500 µm and 100 µm respectively. d) The slices were treated with or without oligomeric Aβ in the presence of PI (red) for 24 h and immunofluorescently stained with TUNEL (green). Increased intensity of PI staining was completely overlapped with TUNEL staining. Scale bar, 100 µm and 40 µm respectively. e) Representative western blots showed oligomeric Aβ induced active caspase-3 after 24 h treatment. Each number represents the mean ± S.E. from three different experiments. *, p<0.05 different from the control.
Oligomeric Aβ Induces Cell Cycle Marker Activation in Hippocampal Slice Cultures

To delineate the potential involvement of neuronal cell cycle re-entry in oligomeric Aβ-induced toxicity in hippocampal slice cultures, I measure DNA replication by measuring of BrdU incorporation in the slice cultures with oligomeric Aβ treatment for 24 h. The number of hippocampal pyramidal CA neurons showing nuclear incorporation of BrdU was significantly increased by oligomeric Aβ treatment (Figure 4-2a) and the BrdU-positive cells were mostly overlapped with neuronal nuclei confirmed by the co-localization of NeuN (Figure 4-2a). In contrast, no BrdU positive neurons were found in non-treated control slice cultures. To extend BrdU incorporation data, I analyzed the expression level of PCNA and cdk4, well established markers of cell cycle activity. Consistent with BrdU incorporation analysis, oligomeric Aβ treatment resulted in the increased expression of PCNA after 24 h incubation (Figure 4-2b). Both cdk4 was also strongly expressed after 24 h incubation, while the expression level of cell cycle inhibitor p27 was decreased (Figure 4-2b). Double immunocytochemistry with PI and an anti-cyclin A antibody demonstrated the significant overlap between cyclin A- and PI-positive cell populations indicating the specific activation of cell cycle in degenerating neuronal cells (Figure 4-2c). Thus, I examined whether oligomeric Aβ treatment increase the phosphorylation and degradation of p27^{kip}. Indeed, oligomeric Aβ treatment significantly increase the phosphorylated p27^{kip} and the degradation of p27^{kip} in slice cultures (Figure 4-2d, e). Taken together, these data strongly indicate that oligomeric Aβ induces
neurons to progress through S phase after 24 h treatment via $p27^{Kip}$ phosphorylation and degradation.
Figure 4-2. Treatment with oligomeric Aβ increases cell cycle markers. a) Incorporation of BrdU during DNA replication was visualized using an anti-BrdU antibody (red). The number of BrdU-positive nuclei was dramatically increased in pyramidal CA neurons in oligomeric Aβ treated slice cultures for 24 h. Double staining with anti-NeuN antibody (green) clearly demonstrates that the BrdU-positive cells were neurons. In control slices, virtually no BrdU-positive nuclei were observed. Scale bar, 50 μm. b) Representative western blots showed oligomeric Aβ induced upregulation of PCNA and cdk4, while p27 expression was downregulated in a time dependent manner. Approximately equal loading of each lane was confirmed using actin antibody. c) The slices were treated with oligomeric Aβ for 24 h in the presence of PI (red) and then fixed and immunofluorescently stained with antibody against cyclin A (green). The overlap of increased intensity of PI with cyclin A was observed. Scale bar, 50 μm. d) Slice cultures treated or untreated with oligomeric Aβ for 24 h were analyzed for p27Kip and phospho-p27Kip (Ser-10) protein levels. Oligomeric Aβ induced degradation of p27Kip and increased phospho-p27Kip expression levels. e) The slices were fixed after oligomeric Aβ treatment for 24 h and stained with anti-p27Kip antibody. p27Kip degradation was observed in slices treated with oligomeric Aβ. p27 expression disappears in oligomeric Aβ-treated hippocampal slices. Scale bar, 100 μm. Data are the mean ± S.E. from three different experiments. *, p<0.05 different from the control.
In Chapter 3, I found that oligomeric Aβ induces phosphorylation of NR2B, which leads to excessive influx of Ca\(^{2+}\) into the cell. Therefore, I reasoned that Ca\(^{2+}\) related signaling pathway may be involved in oligomeric Aβ-induced cell cycle and cell death. To delineate the mechanism by which oligomeric Aβ causes neuronal cell death, I examined the potential involvement of CaMKII and ERK signaling pathways. The slice cultures treated with oligomeric Aβ resulted in a time-dependent activation of CaMKII and ERK1/2 (Figure 4-3a and b) and these results are consistent with the hypothesis that oligomeric Aβ-induced neuronal cell death is mediated through the activation of the CaMKII-ERK signaling pathway. Notably, while ERK1/2 activation was activated in 12 h after oligomeric Aβ treatment, CaMKII was activated earlier, in 6 h treatment suggesting a proximal role of CaMKII in ERK1/2. To clarify the relationship between CaMKII and ERK1/2 pathways, I pretreated KN-93, a selective CaMKII inhibitor, before the oligomeric Aβ treatment to block CaMKII activation by oligomeric Aβ. As shown in Figure 4-3c, indeed, KN-93 significantly reduces ERK1/2 activation in a concentration dependent manner. In addition, NMDA antagonist APV prevents the activation of ERK (Figure 4-3d). Thus, these data strongly support that oligomeric Aβ induce CaMKII-ERK activation through NMDA-R.
Figure 4-3. Activation of CaMKII and ERK in response to oligomeric Aβ. a) and b), Representative western blots showed oligomeric Aβ-induced CaMKII and ERK activation in a time-dependent manner under conditions identical to those described for Fig. 2. The lower panels correspond to the quantification of CaMKII or ERK1/2 activation, normalized to the total amount of actin or ERK1/2 respectively. c) Representative western blots showed the effects of CaMKII inhibitor KN-93 on oligomeric Aβ-induced CaMKII activation or ERK1/2 activation in hippocampal slices. Slices were incubated with KN-93 (5, 15 µM) for 1 h at the concentrations indicated and then treated with 5 µM oligomeric Aβ for 24 h. KN-93 pretreatment attenuated the activation of CaMKII and ERK in concentration dependent manner. d) APV (NMDA antagonist) pretreatment inhibits ERK activation. Data are the mean ± S.E. from four different experiments. *, p<0.05 different from the control.
The inhibition of ERK Suppresses the Neuronal Cell Cycle Re-entry and Neuronal Cell Death Caused by Oligomeric Aβ

Since p27Kip is a proximal regulator of cell cycle progression, I reasoned that the increase of ERK1/2 activity (Figure 4-4a) by oligomeric Aβ may increase the phosphorylation of p27Kip and subsequent degradation, and then initiate cell cycle re-entry. To confirm my hypothesis that ERK cascade may cause p27Kip degradation, I analyzed the degradation of p27Kip with pretreatment of U0126, a specific inhibitor for ERK. Consistent with my hypothesis, the treatment of U0126 significantly suppressed oligomeric Aβ mediated activation of ERK1/2 (Figure 4-4a) and inhibited the degradation of p27Kip in the concentration dependent manner. I also confirmed that the treatment of U0126, flavopiridol or KN-93 inhibited the induction of cyclin A and PCNA by oligomeric Aβ, suggesting ERK-mediated p27Kip degradation is an upstream factor of cell cycle re-entry by oligomeric Aβ (Figure 4-4b). Flavopiridol did not make an effect on oligomeric Aβ -induced ERK activation (data not shown).

To further investigate whether the cell cycle re-entry contributes to the neuronal cell death by the treatment of oligomeric Aβ, I examine the oligomeric Aβ-induced neuronal cell death with pretreatment of the inhibitor for ERK, cell cycle or CaMKII. I found that the treatment of U0126 significantly reduced oligomeric Aβ-mediated toxicity (Figure 4-5a). Flavopiridol or KN-93 also significantly reduced the neuronal toxicity of oligomeric Aβ (Figure 4-5a). I also
confirmed by Nissl staining that each treatment prevents oligomeric Aβ-induced neuronal loss (Figure 4-5b). In addition, these inhibitors suppressed the induction of active caspase-3 (Figure 4-5c). These data suggested that ERK mediates the initiation of cell cycle re-entry by oligomeric Aβ and cell cycle re-entry is an essential mechanism of oligomeric Aβ-induced neuronal cell death in slice cultures.
Figure 4-4. U0126 or KN-93 suppresses p27Kip degradation and cell cycle activation in response to oligomeric Aβ. a) The effect of ERK inhibitor U0126 on oligomeric Aβ-induced p27Kip degradation. Slices were treated with U0126 (2, 5 µM) at the concentration indicated for 1 h before the incubation with oligomeric Aβ for 24 h. U0126 attenuated oligomeric Aβ-induced ERK activation and p27 degradation in the concentration manner. b) The effect of U0126 (5 µM), flavopiridol (1 µM) or KN-93 (15 µM) on oligomeric Aβ-induced cell cycle proteins. Their pretreatment suppressed the increased expression of cell cycle protein induced by oligomeric Aβ. Each number represents the mean ± S.E. from four different experiments. *, p<0.05 different from the oligomeric Aβ-treated samples.
Figure 4-5. Effects of inhibitor for ERK, cell cycle or CaMKII on oligomeric Aβ-induced toxicity. a) Hippocampal slices were incubated with U0126 (5 µM), flavopiridol (1 µM) or KN-93 (15 µM) for 1 h and then treated with 5 µM oligomeric Aβ in the presence of PI for 24 h. PI staining was analyzed with microscopy to determine the effects of these pharmacological agents on oligomeric Aβ-induced toxicity. U0126, flavopiridol and KN-93 significantly inhibited oligomeric Aβ-induced neuronal cell death. b) Nissl staining shows that oligomeric Aβ-induced neuronal cell loss is prevented by each inhibitor pretreatment. c) The effect of U0126, flavopiridol or KN-93 on the expression of active caspase-3 after oligomeric Aβ 24 h treatment. Pretreatment of either drug suppressed the oligomeric Aβ-induced caspase-3 expression. Data are the mean ± S.E. from three different experiments. *, p<0.05 different from the control, #, p<0.05 different from oligomeric Aβ treatment. Scale bar, 500 µm.
The well-known substrates for Cdk4/6 are members of the retinoblastoma protein (pRb) family. Phosphorylation of Rb members by Cdks results in release and activation of the transcription factor E2F, which then activates genes required for S phase transition. Additionally, the role of Rb and E2F in control of apoptosis has also been suggested [113]. I found that oligomeric Aβ induces phospho-Rb after 24 hrs treatment. I also found that oligomeric Aβ induce a tumor suppressor protein, p53 accumulation. It is thus likely that the transcription factor activation may be downstream of neuronal cell cycle re-entry and may activate apoptotic related genes. In addition, by immunoblot analysis with γ-H2AX antibodies, I detected an increase in γ-H2AX following oligomeric Aβ treatment (Fig. 4-6a). Phosphorylation of H2AX (γ-H2AX) is an early sign of DNA damage induced by stalling replication forks. Importantly, inhibitor of cell cycle, ERK or CaMKII prevents p53 and γH2AX accumulation (Fig. 4-6b), suggesting that oligomeric Aβ dependent DNA damage and p53 accumulation may be due to inappropriate DNA synthesis.

Oligomeric Aβ induced accumulation of phospho-Rb, γ-H2AX and p53

The well-known substrates for Cdk4/6 are members of the retinoblastoma protein (pRb) family. Phosphorylation of Rb members by Cdks results in release and activation of the transcription factor E2F, which then activates genes required for S phase transition. Additionally, the role of Rb and E2F in control of apoptosis has also been suggested [113]. I found that oligomeric Aβ induces phospho-Rb after 24 hrs treatment. I also found that oligomeric Aβ induce a tumor suppressor protein, p53 accumulation. It is thus likely that the transcription factor activation may be downstream of neuronal cell cycle re-entry and may activate apoptotic related genes. In addition, by immunoblot analysis with γ-H2AX antibodies, I detected an increase in γ-H2AX following oligomeric Aβ treatment (Fig. 4-6a). Phosphorylation of H2AX (γ-H2AX) is an early sign of DNA damage induced by stalling replication forks. Importantly, inhibitor of cell cycle, ERK or CaMKII prevents p53 and γH2AX accumulation (Fig. 4-6b), suggesting that oligomeric Aβ dependent DNA damage and p53 accumulation may be due to inappropriate DNA synthesis.
Figure 4-6. Oligomeric Aβ induces accumulation of phosphorylated retinoblastoma, p53, and γH2Ax. a) Representative western blots showing oligomeric Aβ-induced phosphorylation of retinoblastoma, p53, and γH2Ax. Approximately equal loading of each lane was confirmed using actin. b) Pretreatment with flavopiridol, U0126, or KN-93 inhibits the accumulation of p53 and γH2Ax.
E2F-1 deficiency protect oligomeric Aβ-induced neuronal cell death

I showed that pharmacological cell cycle inhibitor, flavopiridol, prevented oligomeric Aβ-induced cell death in hippocampal slice cultures (Fig. 4-5). I then tested, genetically, whether cell-cycle activation is upstream of DNA damage and neuronal cell death induced by oligomeric Aβ in the slice culture model. E2F induction is known to be a bottleneck event upstream of PCNA expression and DNA replication [114]. E2F-1 and E2F-4 are the most abundant E2F family members in the brain, but E2F-1, unlike E2F-4, is predominately regulated by p-pRb. Furthermore, E2F-1 has been shown to be the E2F family member that most potently sensitizes cells for apoptosis [115]. I hypothesize that if cell cycle re-entry induces apoptosis pathways in oligomeric Aβ-treated slices, E2F-1 may play a role in the neuronal cell death. I analyzed the cell death in hippocampal slice culture from E2F-1 knockout mouse. Interestingly, E2F-1 knockout significantly suppressed oligomeric Aβ-induced neuronal cell death (Fig 4-7a). Importantly, the knockout prevents the oligomeric Aβ-induced upregulation of cell cycle protein and γH2Ax expression (Fig. 4-7b). Thus, this data suggest that E2F-1 signaling is upstream of DNA damage and cell death in this model.

ATM deficiency protect oligomeric Aβ-induced neuronal cell death

The central proteins of the DNA damage response are ATM and ATR [116]. While ATM and ATR share most downstream effectors, the ATM-Chk2 pathway is primarily activated by DNA damage. I hypothesized that if activation of the
DNA damage response is responsible for triggering the cell death, inhibition of ATM activity might block oligomeric Aβ-induced apoptosis. To determine the role of the DNA damage response pathway in oligomeric Aβ-induced apoptosis, I use hippocampal slice culture from ATM (-/-) mice. I found that ATM knockout is resistant to oligomeric Aβ-induced neurotoxicity (Fig. 4-7a). Importantly, cell cycle activation induced by oligomeric Aβ is still observed in ATM knockout slice culture (Fig. 4-7c), suggesting that cell cycle re-entry is upstream of ATM response. Thus, ATM (-/-) rescues oligomeric Aβ-induced neuronal cell death, suggesting that the DNA damage response pathway through ATM induces apoptosis.

p53 deficiency protect oligomeric Aβ-induced neuronal cell death

I found oligomeric Aβ induced p53 upregulation (Fig. 4-7a). I therefore tested whether knockout of p53 may prevent oligomeric Aβ-induced neuronal cell death in slice culture model. The knockout of p53 significantly rescues oligomeric Aβ-induced cell death (Fig. 4-7d), and importantly the knockout does not make an effect on cell cycle re-entry and γH2Ax upregulation (Fig. 4-7d). This data suggests that p53 is a downstream of cell cycle re-entry and DNA damage response and plays a role in oligomeric Aβ-induced neuronal cell death.
Figure 4-7. Knockout of E2F1, ATM or p53 inhibits the oligomeric Aβ-induced neuronal cell death. (A), (B) Representative PI staining and Nissl stain shows no neuronal cell death by oligomeric Aβ in hippocampal slice from E2F1 of ATM deficient mouse. (C) Representative western blot data shows that lack of E2F1 prevents cyclin A, PCNA and γH2AX upregulation induced by oligomeric Aβ. (D) Representative western blot data shows that lack of ATM prevents γH2AX and p53 accumulation, but not PCNA and cyclin A upregulation induced by oligomeric Aβ. (E) Knockout of p53 has no effect on cell cycle re-entry and γH2AX upregulation. Data are the mean ± S.E. from four different experiments. *, p<0.05 different from the control. Scale bar, 500 μm.
Oligomeric Aβ intrahippocampal injection causes neuronal cell death in vivo.

At last, to further confirm my finding in slice culture models, I investigated the role of cell cycle re-entry in vivo models. Intrahippocampal injection of Aβ1-42 is known to cause a remarkable memory impairment and neuronal apoptosis in vivo [14]. Furthermore, the injection of soluble oligomeric Aβ disrupts learned behavior [15]. At first, to establish my injection models, I analyzed the effect of oligomeric Aβ in neurons in vivo. I injected oligomeric Aβ (5 nM) into the hippocampus of the mouse brain. HE and NeuN staining revealed a loss of neuron in dentate gyrus (DG) at 20 days after injection (Figure 4-8a and b). TUNEL positive cells were observed in oligomeric Aβ injected animals, but not in Aβ42-1 injected animals (Figure 4-8c). Taken together, these data indicate that oligomeric Aβ induce neuronal cell death through apoptotic pathway.
Figure 4-8. Hippocampal oligomeric Aβ injection induced neuronal cell loss and gliosis in vivo. a) Mice were sacrificed and stained with HE at 20 days after oligomeric Aβ injection. Cell bodies in DG region are reduced. Scale bar, 200 μm. b) NeuN staining showed oligomeric Aβ induced neuronal loss in DG region. Scale bar, 500 μm. c) Hippocampal section was stained with TUNEL. Consistent with neuronal cell loss, apoptotic cells are observed in DG region. Scale bar, 200 μm.
Oligomeric Aβ intrahippocampal injection induces cell cycle re-entry in vivo

To see the involvement of cell cycle re-entry in vivo models, I assessed the expression of cell cycle related proteins seen in slice culture. In control animals, no PCNA or cyclin A expression are found in neurons, but oligomeric Aβ injection induces the expression of PCNA, cyclin A and phospho-Rb in neurons at 10 days after injection (Fig. 4-9a, b and c), indicating that oligomeric Aβ injection into hippocampus induce neuronal cell cycle in vivo. I also found the oligomeric Aβ injection induces astrocytosis around DG region (Fig. 4-9d).

Oligomeric Aβ intrahippocampal injection induces accumulation of γH2Ax and p53 in vivo

I analyzed the accumulation of p53 and γH2Ax in oligomeric Aβ-injected animals. As I expected, I found that oligomeric Aβ induce γH2Ax and p53 in 10 days after oligomeric Aβ-injection in DG regions (Fig. 4-10a and b).
Figure 4-9. Oligomeric Aβ injection induced upregulation of cycle re-entry related proteins in vivo. a) The expression of cyclin A is increased in the nuclei of hippocampal neurons (DG region) from oligomeric Aβ-injected mice, indicated days after the injection. Scale bar, 200 μm. b) The expression of PCNA is increased in the neurons. Double staining with NeuN clearly demonstrates that PCNA is expressed in neuronal cells. Scale bar, 200 μm. c) Immunoreactivity for the S-phase associated markers p-pRb is detected in neuronal cells in oligomeric Aβ-injected mice. Scale bar, 100 μm. d) Immunocytochemistry with an anti-GFAP antibody demonstrates the extensive astrocytosis in the hippocampus in oligomeric Aβ-injected mice. Scale bar, 200 μm.
Figure 4-10. Oligomeric Aβ injection induced upregulation of γH2Aγ and p53 in vivo. a) Immunoreactivity for the DNA double strand breaks marker, γH2Aγ, is increased in DG region at 10 days after injection. b) p53 accumulation is observed in hippocampus from oligomeric Aβ-injected mice at 10 days after injection. Scale bar, 100 μm.
E2F1, ATM or p53 deficiency protect oligomeric Aβ-induced neuronal cell death in vivo

Finally, I tested whether knockout of E2F-1, ATM or p53 in mice could mitigate oligomeric Aβ toxicity in vivo. Indeed, after Oligomeric Aβ hippocampal injection, stereological cell counts demonstrated that there was significantly less neuronal cell loss in E2F-1-/- mice, ATM -/- mice and p53-/- mice than in WT mice (Fig. 4-11a and b). In addition, no TUNEL positive cells were found in E2F-1-/-, ATM-/- and p53-/- mice (Fig. 4-11c). Importantly, ATM-/- and p53-/- mice still show cyclin A upregulation in neurons, but E2F-1 mice has no cyclin A expression (Fig. 4-11d), which suggest that ATM and p53 play a role in cell death pathway on a downstream of oligomeric Aβ-induced cell cycle re-entry. In addition, oligomeric Aβ injection induces γH2Ax in only p53-/- mice. These data indicates that consistent with slice culture models, cell cycle re-entry and DNA damage response may initiate apoptotic pathway through ATM-p53 pathway in oligomeric Aβ-injection model in vivo.
a

Control  WT/AB  E2F1(-/-)/AB  ATM (-/-)/AB  p53 (-/-)/AB

b

Control  WT/AB  E2F1(-/-)/AB  ATM (-/-)/AB  p53 (-/-)/AB

NeuN

c

WT/AB  E2F1 (-/-)/AB  ATM (-/-)/AB  p53 (-/-)/AB

TUNEL

TUNEL + DAPI
Figure 4-11. Knockout of E2F-1, ATM or p53 provides protection against oligomeric Aβ hippocampal injection in vivo. a) and b) Representative photomicrographs (HE and NeuN staining) in the DG region shows that E2F-1-/-, ATM-/- or p53-/- mice are resistant to oligomeric Aβ-induced cell death at 20 days after Oligomeric Aβ-injection. c) No TUNEL positive cells are found in E2F1-/-, ATM-/- or p53-/- mice. d) Immunohistochemistry data shows that knockout of E2F1 suppress cyclin A upregulation, but knockout of ATM and p53 has no effect on cell cycle re-entry at 10 days after Oligomeric Aβ-injection. e) Immunocytochemistry with an anti-γH2Ax antibody demonstrates knockout of E2F1 and ATM prevent γH2Ax accumulation in the hippocampus from oligomeric Aβ-injected mice. Scale bar, 100 μm.
Discussion

Although accumulated evidences suggest the importance of cell cycle re-entry in the pathogenesis of AD [38, 102, 117], the signaling mechanism(s) controlling the cell cycle re-entry is still unclear. In the present study, I have shown that neuronal cell cycle re-entry plays a critical role in the neurotoxicity induced by oligomeric Aβ in mouse hippocampal slice cultures and in vivo Aβ injection model. I also verified that this oligomeric Aβ-induced cell cycle re-entry is mediated through the activation of the CaMKII-ERK pathways, which results in degradation of p27Kip and subsequent cell cycle re-entry. Furthermore, I found that the inappropriate cell cycle induces DNA damage by a replication stress and lead to apoptotic signaling through ATM-p53.

Multiple lines of evidence in this study support my conclusion. First, I confirmed that oligomeric Aβ induced neuronal specific cell death, which is a caspase-3 dependent apoptosis. Second, I observed the activation of ERK1/2 and CaMKII in the slice cultures exposed to oligomeric Aβ and attenuation of ERK1/2 activation by an inhibitor for CaMKII. Third, I revealed that oligomeric Aβ caused neuronal cell cycle re-entry after 24 h treatment evidenced by BrdU incorporation and the increased expression of the S-phase cell cycle proteins such as PCNA, cdk4 in vitro and in vivo. Fourth, the inhibition of ERK, CaMKII activity prevented the induction of cell cycle re-entry by blocking the degradation of p27Kip. Fifth, the inhibition of ERK, cell cycle or CaMKII significantly attenuated the oligomeric Aβ induced-neuronal cell death. Finally, knockout of E2F1, ATM or p53 prevents oligomeric Aβ-induced neuronal cell death in vitro.
and *in vivo*. Taken together, I concluded that the neuronal cell cycle plays an essential role in oligomeric Aβ-induced neuronal cell death, which occurs through CaMKII-ERK1/2 signaling cascade.

It has been shown that the inhibition of CaMKII induces cell cycle arrest and regulates cell cycle progression of tumor cells [105]. It is also known that inhibition of CaMKII represses the activation of many important signal transduction pathways, such as protein kinase C, ERK, p38, or c-Jun N-terminal kinase in smooth muscle cells [118] and cultured rat cortical neurons [119]. Previous studies, however, showed that JNK inhibitor or p38 inhibitor does not have any effect on oligomeric Aβ-induced neuronal cell death [52], and in this study, I found that ERK inhibitor significantly prevented the cell death. I also found that activated CaMKII increased the phosphorylation of ERK1/2 and that the inhibition of ERK1/2 prevented the degradation of p27^Kip^ in the slice cultures exposed to oligomeric Aβ. These data strongly suggest that p27^Kip^ degradation and phosphorylation is dependent on the ERK signaling pathway and that the CaMKII-ERK1/2 cascade is a key regulator in cell cycle re-entry induced by oligomeric Aβ. The previous studies indicated that ERK1/2, but not CaMKII, can phosphorylate the p27^Kip^ directly *in vitro* and in cell culture model [106, 120], supporting the regulation of p27^Kip^ activity by ERK in my model system.

Consistently, I observed that the cell cycle-like events in hippocampal slice culture are not a consequence of apoptosis associated caspase activity. Rather, E2F-1 seems to act as an upstream of DNA damage and neuronal cell death. *In
vitro and in vivo data show knockout of E2F-1 significantly protected oligomeric Aβ-induced neuronal loss. E2F-1 was originally proposed to be the only member of the E2F family that induces apoptosis, although recent data suggest that other E2F proteins trigger apoptosis as well [114]. Nonetheless, E2F-1 appears to be a crucial upstream mediator of oligomeric Aβ-induced neurodegeneration.

DNA damage occurs in response to toxic chemicals, as a byproduct of normal metabolism through the generation of reactive oxygen species, or through errors in DNA replication, such as DNA replication fork stalling. My results demonstrated that oligomeric Aβ causes DNA damage as seen by increases in γ-H2AX. However, the disappearance of γ-H2AX in E2F1 knockout and cell cycle inhibitor treatment suggests that the DNA damage may be due to replication stress rather than more traditional DNA breaks, such as caused by DNA damaging agents like camptothecin. In addition to E2F1, I also found knockout of ATM or p53 are protective against oligomeric Aβ-induced neuronal cell death in vitro and in vivo. While several papers have suggested that DNA damage itself is the causative agent of cell cycle reactivation and DNA synthesis [121], in my system, I found that blocking the DNA damage response pathway with ATM knockout mouse or p53 knockout, did not block cell cycle re-entry, as seen by PCNA and cyclin A upregulation. Neurons still entered S phase, but in the absence of the protective effect of the DNA damage response, apoptosis was prevented. The results from knockout studies suggest that oligomeric Aβ-induced apoptosis is due to S phase entry, and subsequent DNA replication stalling might be the cause of DNA damage, activating the DNA damage response, such as ATM. The
activation of the DNA damage response pathway was responsible for oligomeric Aβ-dependent neuronal cell death, as inhibition of this pathway blocked the induction of apoptosis, suggesting that it may play an important role in inducing apoptotic pathway.

Collectively, I propose here a mechanistic model by which oligomeric Aβ causes cell cycle progression in hippocampal slice cultures (Figure 4-12). In this model, oligomeric Aβ increases intracellular Ca²⁺ [122] through NMDA receptor and this event activates CaMKII, which subsequently activates the ERK1/2 pathway. Next, activated ERK1/2 either directly or indirectly phosphorylates p27^Kip, allowing p27^Kip to binding the nuclear exporter CRM1 and subsequent p27 degradation [123]. As a result, the cell cycle is re-activated and induced DNA damage via replication stress, following apoptotic pathways via ATM-p53 accumulation, which results in subsequent neuronal cell death in hippocampal slices exposed to oligomeric Aβ. Our recent study showed that cell cycle re-entry in fully developed adult neurons results in neuronal cell death, gliosis, and cognitive deficits in transgenic mice [42], suggesting that the activation of cell cycle in adult neurons is detrimental and supporting the causal role of cell cycle re-entry in AD pathogenesis. Taken together with the results presented here, this data strengthens my hypothesis that cell cycle re-entry is an important mechanism for the neurodegeneration of AD.
Figure 4-12. A schematic of a potential pathway underlining the effects of oligomeric Aβ on neuronal cell death and neuronal cell cycle in vitro and in vivo. Oligomeric Aβ cause the intracellular Ca$^{2+}$ influx in hippocampus through NMDA receptor. Activation of CaMKII phosphorylates MEK, leading to ERK activation. The phosphorylation of p27$^{kip}$ by ERK is enhanced directly or indirectly. Hyperphosphorylation of p27$^{kip}$ results in p27$^{kip}$ degradation by activated calpain, which is regulated by ERK pathway and therefore leads to degradation of p27$^{kip}$. The cell cycle re-entry causes DNA damage by DNA replication stress and induces DNA damage response protein ATM. ATM induces the p53 aggregation and alteration of apoptotic related proteins levels, which results in neuronal cell death via caspase-3 activation.
CHAPTER 5; Inhibition of Bax Protects Neuronal Cells from Oligomeric Aβ-induced Toxicity
Abstract

Although oligomeric β-amyloid (Aβ) has been suggested to play an important role in Alzheimer’s disease (AD), the mechanism(s) of how Aβ induces neuronal death has not been fully identified. The balance of pro- and anti-apoptotic Bcl-2 family proteins (e.g. Bcl-2 and Bcl-w vs. Bad, Bim, and Bax) has been known to play a role in neuronal cell death and, importantly, expression levels of these proteins are reportedly altered in the vulnerable neurons in AD. However, the roles of apoptotic proteins in oligomeric Aβ-induced cell death remain unclear in vivo or in more physiologically relevant models. In addition, no study to date has examined whether Bax is required for the toxicity of oligomeric Aβ. Here, I found that treatment with oligomeric Aβ increased Bim levels but decreased Bcl-2 levels, leading to the activation of Bax and neuronal cell death in hippocampal slice culture and in vivo. Furthermore, the inhibition of Bax activity either by Bax inhibiting peptide or bax gene knockout significantly prevented oligomeric Aβ-induced neuronal cell death. These findings are first to demonstrate that Bax plays an essential role in oligomeric Aβ-induced neuronal cell death, and that targeting of Bax may be a therapeutic approach for AD.

Introduction

Alzheimer disease (AD) is the most common neurodegenerative disorder and β-amyloid (Aβ) has been suggested to play a critical role in the pathogenesis of
AD. It has been shown that oligomeric Aβ is a main Aβ species inducing neurodegeneration in AD, yet the molecular mechanism(s) of its neurotoxicity remains elusive [62]. At present, it is reported that oligomeric Aβ induces apoptotic neuronal death in the rat and mouse neurons in vitro and in vivo [124, 125].

Previous studies found that expression levels of Bcl-2 family proteins, such as Bax, Bak, Bad, Bcl-2, Bim, Bcl-w and Bcl-x are altered in the vulnerable neurons in AD [126]. Bcl-2 family is structurally defined by the presence of up to four conserved ‘BCL-2 homology’ (BH) domains. The family proteins are key regulators of evolutionally conserved pathway of apoptosis [127, 128]. Bax and Bak belong to the multi-BH domains pro-apoptotic subfamily which promotes apoptosis by translocating into the mitochondrial membrane and facilitating cytochrome c release, while Bcl-2 and Bcl-XL belong to the prosurvival subfamily which prevents apoptotic death in multiple cell types including neuron [129]. It is hypothesized that the BH3-only proteins, such as Bim, Bid, Puma, Noxa and Bad, induce the activation of Bax and Bak, either directly or indirectly by inactivating the prosurvival Bcl-2 proteins. In the presence of apoptotic stimuli, Bax is known to change its conformation. Specifically, as an early step of Bax activation, the N-terminus exposure is considered a prerequisite for membrane insertion of Bax at mitochondria and multimerization of Bax [130, 131]. Ku70, a DNA repair factor, can prevent the conformational change by binding with Bax in the cytosol, which leads to the inhibition of Bax-mediated cell death. Previously, a cell permeable Bax-inhibiting peptide (BIP), designed to induce the Bax binding
domain of Ku70, was found to rescue cells from Bax-mediated cell death [132-134].

Notably, overexpression of anti-apoptotic proteins, Bcl-w, or genetic ablation of a proapoptotic effector, Bim, significantly protected neurons against fibrillar Aβ-induced apoptosis in neuroblastoma cell lines and primary neuron culture [135, 136]. Giovanni et al. also reported that fibrillar Aβ-induced cell death is dependent on Bax in primary neuron culture [137]. However, these results have never been confirmed in vivo or in a more physiologically relevant model and all the previous studies examined the toxicity of fibrillar Aβ, not oligomeric Aβ. Therefore, to advance our understanding of the involvement of Bcl-2 protein family as the major mechanism of oligomeric Aβ–induced neuronal cell death, in this study, I examined the effect of oligomeric Aβ on the regulation of Bcl-2/Bim/Bax and its functional importance in neuronal cell death in the organotypic hippocampal culture and mouse model for Aβ toxicity.

**Materials and Methods**

*Materials*

Anti-β-actin antibody was obtained from Millipore and anti-cleaved-caspase-3 antibody was obtained from Cell Signaling Technology. Aβ peptide (Aβ1-42) and reverse control peptide (Aβ42-1) were purchased from ANA spec. Propidium Iodide (PI) and other chemicals were obtained from Sigma.
Preparation of oligomeric Aβ

Soluble oligomeric Aβ was prepared from synthetic peptide according to a previous paper.[49] Briefly, 1 mg of Aβ₁₋₄₂ peptide was dissolved in 120 µl of hexafluoroisopropanol for 60 min at room temperature, and placed back on ice for 5-10 min. After evaporation of hexafluoroisopropanol overnight in the hood at room temperature, the peptide was dissolved in 40 µl of fresh anhydrous DMSO, and further diluted to 5 mM stock solution. The stock peptide solution was then incubated for 24 h at 4°C, and centrifuged at 14,000 g for 10 min at 4°C. Supernatant was used as oligomeric Aβ. Before I treated slice culture with oligomeric Aβ, the oligomers were incubated at room temperature for 20 h.

Mouse strains

Bax knockout (KO) mice (strain name: B6.129X1-Bax^tm1Sjk/J.) were purchased from The Jackson Lab. This Bax KO mouse has C57BL/6 genetic background. Bax KO mice were crossed with wild type C57BL/6 mice, and bax+/- mouse colony was generated. Bax KO as well as wild type (WT) mice were generated by crossing bax+/- mice in our mouse colony. Each experiment used a set of Bax KO and WT mice obtained from the same parents to minimize variation caused by genetic differences among lines.

Preparation of organotypic hippocampal slice cultures

Organotypic hippocampal slice cultures were prepared as described previously [50]. Briefly, hippocampal slice cultures were prepared from 7-10 day-old mouse
pups. Slices were cut at 400 µm on a Mcllwain tissue chopper, transferred to Millicell (Millipore Corp.) membrane inserts (0.4 µm), and placed in 6-well culture plates. The culture medium was consisted of basal Eagles medium with Earle’s balanced salt solution, 20% heat-inactivated horse serum, enriched with 5.6 mM glucose. The medium was changed every other day. The effect of oligomeric Aβ (500 nM) was tested in the slices which had been maintained for 11-14 days in vitro. Oligomeric Aβ or reversed sequence of Aβ1-42 control peptide (Aβ42-1) was added to cultures in serum free medium and, after the treatment, the hippocampal slices were rinsed twice in ice cold PBS, and then harvested by removing the Millicell membrane insert.

Assessment of neuronal cell death in organotypic hippocampal slice cultures

To determine neuronal cell death in the hippocampal slices, propidium iodide (PI) (5 µg/ml) was added to the slice culture medium. Images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss). Fluorescent intensity was measured using Image J. Nissl staining was also performed for routine histochemical and morphological analyses.

Protein extraction and immunoblot analysis

After oligomeric Aβ treatment, the slices were rinsed twice with ice-cold PBS and then lysed in ice-cold cell lysis buffer (Cell signaling). The protein concentration was determined by BCA assay (Pierce). The extracted proteins were separated on 10 or 12% SDS-polyacrylamide gel and transferred onto
polyvinylidene difluoride membranes. The blots were blocked with 10% non-fat milk in TBS-T for 1 h at room temperature, and then treated with primary antibodies diluted with 1% non-fat milk and incubated overnight at 4°C. The following antibodies were used for immunoblot analysis: anti-caspase-3 (1:1000; Santa Cruz), anti-Bim (1:1000; Stressgen), anti-Bcl-2 (1:1000; Stressgen) and 6A7 (conformational specific Bax antibody) (1:1000; BD Pharmingen).

**Intrahippocampal injection of oligomeric Aβ**

C57BL6/J mice (Jackson Laboratories; 2-3 month old) were anaesthetized with pentobarbital and placed in a stereotaxic frame. Injection was made using a 10 μl microsyringe (Hamilton). A volume of 1 μl of oligomer Aβ dissolved at 50 μM in PBS was injected into the left hippocampus. Control animals were prepared identically and injected with the same volume and concentration of Aβ42 in PBS. Injections were made at stereotaxic coordinates of Bregma; antereoposterior (AP) = 2.3mm, mediolateral (ML) = 2.5mm, dorsoventral (DV) = -2.5mm according to a previous report [138]. This corresponds to a site in the dorsal hippocampus in the apical dendritic zones of the CA1 region near the hippocampal fissure. Mice were sacrificed 10 or 20 days after injection, brains dissected out, fixed in 10% buffered formalin and paraffin embedded. For brain tissue sections, six-μm-thick serial sections were cut, mounted onto slides, and rehydrated according to standard protocols.

**Immunocytochemistry**
Cultured hippocampal slices were rinsed with ice-cold PBS once and fixed for 2 h with 4% paraformaldehyde in 0.1 M phosphate buffer [139]. After washing with PBS, sections were permeabilized overnight with PBS containing 0.2% Triton X-100. At the end of the permeabilization blocking solution, 10% normal goat serum in PBS was applied for 4 h at room temperature. After washing with PBS, primary antibody was added and incubated for 24-48 h at 4°C. After thorough washing of the sections in PBS, a secondary antibody labeled with either Alexa Fluor 488 or 568 (1:300) was added and incubated for 4 h at room temperature. All of the experiments contained at least one sample incubated without a primary antibody to exclude nonspecific signal. Nuclei were visualized with DAPI. Images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss). Images were then analyzed with the Axiovision software (Zeiss).

Terminal dUTP Nick-End Labeling (TUNEL) Analysis

Detection of 3’-OH termini of DNA strand breaks was performed using in situ cell death detection kit (Roche). Briefly, the tissue sections were treated with proteinase K (20 µg/ml in 10 mmol/l Tris-HCl, pH 7.4) for 30 minutes at 37°C after rehydration. After rinsing with PBS, TUNEL reaction mixture containing terminal deoxynucleotidyl transferase and fluorescence-labeled nucleotide was applied for 1 hour at 37°C. The samples were then washed and mounted using Aquamount (Southern Biotech).
**Statistical analysis**

Data were expressed as the means ± S.E.; the number of independent experiments is indicated in the corresponding figure legend. Differences between groups were examined for statistical significance using one-way analysis of variance with an unpaired Students t-test. A $p$ value less than 0.05 indicated a statistically significant difference.

**Results**

*Oligomeric Aβ induces Bim upregulation and Bcl-2 downregulation in hippocampal slice culture*

Time-dependent changes of the levels of Bax, Bcl-2, and Bim were examined in oligomeric Aβ-treated brain slices. Oligomeric Aβ increased the expression of Bim but decreased the level of Bcl-2 (Figure 5-1a). However, the level of Bax was not affected by oligomeric Aβ (Figure 5-1a). Since the N-terminus exposure is an early step of Bax activation that occurs in the cytosol, I analyzed this conformational change of Bax with a monoclonal antibody (6A7) recognizing the epitope in the N-terminus of Bax [131]. While the level of Bax expression was not affected by oligomeric Aβ, the number of 6A7 positive cells were significantly increased in oligomeric Aβ treated slice cultures (Figure 5-1b), suggesting that oligomeric Aβ induces Bax activation.
Figure 5-1. Oligomeric Aβ differentially regulates Bim, Bcl-2 and Bax. a) Representative western blots showed oligomeric Aβ induced upregulation of Bim and downregulation of Bcl-2 in a time dependent manner. Bax levels were not changed by oligomeric Aβ treatment. Actin was used as internal loading control. The indicated comparisons are significant at *p<0.05, n=4. b) Immunocytochemistry analysis demonstrated the increased number of positive cells stained with 6A7 antibody, which specifically detect the active form of Bax, 24 hrs after oligomeric Aβ treatment in hippocampal slice cultures. Conversely, control peptide (Aβ42-1) did not induce the active form of Bax (green: 6A7, blue: DAPI). Scale bar, 100 μm.
Ablation of Bax reduces oligomeric Aβ-induced neuronal cell death in hippocampal slice culture

To further determine the functional role of Bax in oligomeric Aβ induced neuronal cell death, hippocampal slice culture prepared from wild type and \( bax^{-/-} \) mice (Figure 5-2a and b) were used. Slices were treated with oligomeric Aβ in the presence of propidium iodide (PI) which penetrates cell membranes of dead or dying cells, and is widely used for evaluation of cell death. The number of PI positive cells was significantly increased in oligomeric Aβ-treated slices after 48 hrs of the treatment, indicating that oligomeric Aβ induces neuronal cell death in hippocampal slice culture. However, the neuronal cell death induced by oligomeric Aβ was dramatically reduced in the slice culture from \( bax^{-/-} \) mice (Figure 5-2c). Nissl staining analysis further confirmed neuronal cell death by oligomeric Aβ in wild type mouse slice culture but not in \( Bax^{-/-} \) mouse slice culture (Figure 5-2d). These results indicate that Bax is a critical mediator for the neurotoxicity induced by oligomeric Aβ.
**Figure 5-2. Bax<sup>-/-</sup> neurons are resistant to oligomeric Aβ neurotoxicity.**

a) DNA was extracted from bax<sup>+/+</sup> and bax<sup>-/-</sup> mice and each genotype was identified by PCR with the primer sets specifically detecting each genotype as described in the previous study. bax<sup>+/+</sup> yields a 304 bp PCR product and bax<sup>-/-</sup> yields a 507 bp PCR product.

b) Total protein (20 μg) from whole brain was analyzed by immunoblot with anti-Bax antibody. Immunoblot analysis showed Bax in bax<sup>+/+</sup> mouse samples at the expected molecular weight of 20 kDa, and no expression of Bax in bax<sup>-/-</sup> samples.

c) Hippocampal slice culture were treated with oligomeric Aβ (500 nM) in the presence of PI (red) for 48 h. Representative data showed oligomeric Aβ-induced PI staining was significantly reduced in the slice cultures from bax<sup>-/-</sup> mice compared to bax<sup>+/+</sup> mice. The PI staining were quantitatively analyzed (n=5). Control Aβ<sub>42-1</sub> peptide had no effect on PI staining.

Scale bar, 500 μm. *p < 0.05.

d) Neuronal cell loss by oligomeric Aβ (arrows) was significantly reduced in the hippocampal slice cultures from bax<sup>-/-</sup> mice. Scale bar, 500 μm.
Intra-hippocampal injection of oligomeric Aβ increases Bim expression and active forms of Bax

To further examine the involvement of pro-apoptotic proteins in oligomeric Aβ-induced cell death, I determined the change of the levels of Bim and the active form of Bax in vivo. I injected oligomeric Aβ into the hippocampus and assessed its neurotoxicity. Aβ42-1 was used as a control. Oligomeric Aβ injection into the wild type mouse hippocampus induced neuronal cell death in 20 days after injection (Figure 5-3a). The number of TUNEL positive neurons was significantly increased by Aβ injection (Figure 5-3b). To see the involvement of Bim and Bax, I measured the level of Bim and active form of Bax and found that the level of Bim was dramatically increased as early as 10 days after oligomeric Aβ injection (Figure 5-3c). The number of neurons containing the active form of Bax recognized by 6A7 antibody was also dramatically increased by Aβ injection (Figure 5-3d). These data further support that both Bim and Bax play roles in oligomeric Aβ-induced neuronal cell death.

Bax-inhibiting peptide (BIP) suppresses neuronal cell death and Bax activation induced by oligomeric Aβ in hippocampal slice culture

In this study, in addition to utilizing Bax knockout mice, I utilized Bax-inhibiting peptide (BIP) to examine the role of Bax in oligomeric Aβ-induced neurotoxicity. BIP used in this study consists of five amino acids, VPTLK, a
sequence which is known to inhibit Bax activation [132, 133, 140]. A mutated (scrambled) peptide, KLVPT, which does not bind Bax but has the same cell permeability, was used as a negative control. Both peptides were tagged with fluorescein isothiocyanate (FITC) so that intracellular delivery can be tracked by FITC signal. I first treated hippocampal slices with each peptide and analyzed green fluorescence to confirm their cell permeability and confirmed that BIP and the control peptide equally penetrated neuronal cells after 24 hrs treatment (Figure 5-4a). To determine whether BIP is able to suppress oligomeric Aβ-induced neuronal cell death in slice culture, either BIP or control peptide were co-applied with oligomeric Aβ. After 24 hrs treatment of oligomeric Aβ and the peptide, the treatment of BIP significantly prevented PI staining, while the control peptide did not affect the level of PI staining (Figure 5-4b), suggesting BIP specifically blocks neuronal cell death induced by oligomeric Aβ. Consistent with the PI staining results, both Nissl staining and caspase-3 immunoblot analyses also showed that BIP significantly suppressed oligomeric Aβ-induced neuronal loss and caspase-3 activation, but control peptide failed to prevent neuronal cell death (Figure 5-4c and d). I also found that the treatment of BIP suppresses oligomeric Aβ-induced conformational change of Bax (Figure 5-5). These results further support the hypothesis that the activation of Bax by oligomeric Aβ is an essential mechanism of oligomeric Aβ-induced neuronal cell death.
Figure 5-3. Intra-hippocampal injection of oligomeric Aβ upregulates Bim and activates Bax. C57BL/6J mice were sacrificed and the levels of Bim and active Bax were analyzed at 10 or 20 days after oligomeric Aβ injection. a) At 20 days after oligomeric Aβ injection, neuronal cell loss in hippocampus was evident in Aβ injected mice in H & E staining, but not in control peptide injected mice. The region of neuronal cell loss is indicated by dotted lines. Scale bar, 100 μm. b) The number of TUNEL positive cells in hippocampus was dramatically increased after oligomeric Aβ injection while virtually no TUNEL positive cell (green) was detected in control peptide injected hippocampus tissues. Blue: DAPI. Scale bar, 200 μm. c) Immunoreactivity for Bim (green) was increased in hippocampus at 10 days after the oligomeric Aβ injection. Scale bar, 100 μm. d) The number of active Bax positive cells detected by 6A7 antibody (red) was dramatically increased at 10 days after the oligomeric Aβ injection. Scale bar, 100 μm compared to control peptide injected mice.
Figure 5-4. Bax inhibiting peptide (BIP) suppresses neuronal cell death induced by oligomeric Aβ. Either BIP (VPTLK) or control peptide (KLPVT) was added to hippocampal slice cultures at the same time with oligomeric Aβ to examine its neuroprotective effect. a) Cell permeability of both peptides was confirmed by green fluorescence in hippocampal slice cultures. Strong green fluorescence of both BIP and negative control peptide was found at 24 hrs after the peptide treatment indicating both peptides are penetrated to neurons. b) The intensity of PI in slices treated with oligomeric Aβ for 48 h was quantified as a marker of cell death. BIP significantly prevented oligomeric Aβ-induced PI staining (n=5). Scale bar, 500 μm. *p<0.05 and **p<0.01 versus control, #p<0.05 versus Aβ only (-). c) Nissl staining demonstrated that the treatment of BIP significantly prevented neuronal cell loss induced by oligomeric Aβ. Arrows indicate the region of neuronal cell loss. (d) BIP suppressed the activation of caspase-3 (cleaved caspase-3) induced by oligomeric Aβ (n=4). **p<0.01 versus control, #p<0.05 versus Aβ only (-).
Figure 5-5. BIP prevents the conformational change of Bax induced by oligomeric Aβ. BIP (VPTLK) was treated to hippocampal slice cultures with oligomeric Aβ for 48 hrs. Aβ$_{42-1}$ was used as a control. The slices were stained with 6A7 antibody for detecting active conformational change of Bax. In the BIP treated slices, the number and intensity of 6A7 positive signal (green) was significantly decreased compared to the slices treated with Aβ only. Scale bar, 100 μm.
Discussion

In this study, I demonstrate that oligomeric Aβ altered the expression levels of Bcl-2, Bim and Bax, and that genetic or pharmacological ablation of Bax activity, suppresses oligomeric Aβ-mediated neurotoxicity in both *ex vivo* and *in vivo*. These results clearly indicate that Bax plays an essential role in the induction of neuronal cell death caused by oligomeric Aβ.

In healthy cells, Bax is located in the cytosol or loosely associated to mitochondria and ER [141]. Bax translocation to the mitochondria, which occurs in cells with apoptotic stresses, is thought to lead to mitochondrial dysfunction and release of cytochrome c and subsequent apoptosis [142]. Before its translocation to mitochondria, Bax changes its conformation, which exposes the N-terminal residues [143]. This conformational change is believed to be necessary for membrane insertion of Bax at mitochondria and multimerization of Bax [144]. The present study demonstrates that oligomeric Aβ induced the N-terminal exposure of Bax in neuron and that the inhibition of this event by BIP rescues neurons from oligomeric Aβ’s neurotoxicity, suggesting the activation of Bax by its conformational change is a key element of oligomeric Aβ-induced neurotoxicity.

While the molecular mechanism(s) of Bax activation has not been clearly defined, multiple pro-apoptotic proteins (e.g., Bim) and anti-apoptotic proteins (e.g., Bcl-2) are known to regulate the activation of Bax thorough heterodimerization [145]. In fact, consistent with the previous findings, my current data demonstrate that the level of Bim is significantly upregulated after
oligomeric Aβ treatment, while anti-apoptotic protein Bcl-2 is downregulated, suggesting that oligomeric Aβ induces neuronal cell death through the alteration of the balance of Bcl-2 family proteins and consequent activation of Bax. Unlike most 'BH3-only' proteins, Bim can interact with all pro-survival Bcl-2 proteins with high affinity, and is one of the few BH3-only proteins that can directly activate Bax [146]. For instance, several in vitro data suggested that the direct physical interaction between Bim and Bax can drive Bax activation [147, 148]. Importantly, the ablation of Bim suppresses Aβ-induced cell death in primary neurons [135] and overexpression of anti-apoptotic proteins (Bcl-w) significantly protected neurons against Aβ-induced apoptosis [136]. Importantly, previous reports suggested that overexpression of Bim in cultured neurons induces a rapid apoptotic death [149] and Bim is highly expressed in AD neurons [135]. Indeed, Bim is also known to induce Bax activation directly or indirectly [150]. Collectively, these data strongly suggest that Bim might be an upstream regulator of Bax activation in oligomeric Aβ-induced neurotoxicity and the pathological role of Bim and Bax in neuronal cell loss in AD.

However, it remains unknown how oligomeric Aβ activates Bim and consequent neuronal cell death. While future study will be required to determine the mechanism, one potential pathway might be c-Jun N-terminal kinase (JNK)-dependent phosphorylation as JNK-mediated phosphorylation promote Bax-dependent apoptosis [151]. Since the activation of JNK by Aβ has been shown [152] and the active form of JNK has been reported to be increased in vulnerable neurons in AD [153], it is plausible that the activation of JNK and subsequent
activation of Bim/Bax-mediated apoptosis pathway might be the mechanism causing neuronal cell death in oligomeric Aβ-treated neurons and AD. Alternately, cyclin-dependent kinase 4 (cdk4) has been suggested as an activator of Bim. Induction of Bim in NGF-deprived neuronal cells requires active cdk4 and consequent re-activation of a series of genes suppressed by E2F [154]. Among the re-activated genes, members of the myb transcription factor family, especially c-myb, seem to play a role in the activation of Bim. In previous studies, the inhibition of cdk by various experimental approaches (i.e., chemical inhibitors, dominant-negative constructs, siRNA) suggest that the role of cdk4 in the induction of Bim and neuronal cell death evoked by Aβ [135, 137, 155]. Given that the elevated expression of cell cycle-related proteins including cdk4 is evident in the vulnerable neurons in AD [40, 156, 157] and Aβ treated neuronal cells [43, 155], an abortive re-entry into the cell cycle might induce neuronal cell death by upregulating Bim/Bax apoptotic pathway. Supporting the causal role of cell cycle re-entry in neuronal cell death, I previously found that dysregulation of cell cycle re-entry results in neurodegeneration in vivo [42].

In conclusion, in this study, I found that bax−/− mice are resistant to oligomeric Aβ-induced neuronal cell death, suggesting the essential role of Bax in neurotoxicity of oligomeric Aβ. Furthermore, I show for the first time that BIP application prevents oligomeric Aβ-induced neuronal cell death, suggesting that BIP and its mimetics may be utilized to mitigate the progress of AD by rescuing neurons from Bax-induced cell death. My study also suggests that oligomeric Aβ directly regulates the activity of Bim, Bcl-2 and Bax in neurons. Taken all
together, it is highly likely that the activation of Bax by the regulation of Bim and Bcl-2 family is crucial for oligomeric Aβ-induced neuronal cell death.
CHAPTER 6: Conclusions
Summary of Results

The purpose of my thesis project was to elucidate the role of cell cycle re-entry in oligomeric Aβ-induced neuronal cell death and to identify the signaling mechanisms induced. I also investigated the role of PrP in the Aβ induced cell death and the neurotoxic molecular mechanisms that operate through PrP<sup>C</sup>.

The first mechanistic insight into a possible role of PrP<sup>C</sup> in Aβ was provided by the Strittmatter group [20]. Although several groups suggested the physical binding of Aβ to PrP<sup>C</sup>, the role of PrP<sup>C</sup> in neuronal cell death remains elusive. In Chapter 2, I showed that Prnp<sup>−/−</sup> mice are resistant to the neurotoxic effect of oligomeric Aβ <i>in vivo</i> and <i>in vitro</i>. Furthermore, application of an anti-PrP<sup>C</sup> antibody or PrP<sup>C</sup> peptide prevents oligomeric Aβ-induced neurotoxicity.

In Chapter 3, I investigated the oligomeric Aβ-induced neurotoxic signaling through PrP<sup>C</sup>. Oligomeric Aβ induces NR2B phosphorylation through Fyn kinase, which is dependent on PrP<sup>C</sup> expression. Treatment with NR2B antagonist, but not NR2D, reduces the neuronal cell death. Knockdown of caveolin-1 diminishes the interaction between PrP<sup>C</sup> and Fyn, and inhibits oligomeric Aβ-induced NR2B phosphorylation. I then found that lack of caveolin-1 blocks oligomeric Aβ-induced neuronal cell death. Taken together, these data demonstrate that caveolin-1 mediates oligomeric Aβ-induced neurotoxic signaling through PrP<sup>C</sup>.

In Chapter 4, I examined the role of cell cycle re-entry in oligomeric Aβ-induced neuronal cell death. Oligomeric Aβ caused neuronal cell cycle re-entry after 24 h treatment evidenced by BrdU incorporation and the increased
expression of the S-phase cell cycle proteins such as PCNA and cdk4 \textit{in vitro} and \textit{in vivo}. I found that inhibition of ERK or CaMKII activity prevented the induction of cell cycle re-entry by blocking the degradation of p27Kip. The inhibition of ERK, cell cycle, or CaMKII significantly attenuated the oligomeric Aβ induced-neuronal cell death. Finally, knockout of E2F1, ATM or p53 prevents oligomeric Aβ-induced neuronal cell death \textit{in vitro} and \textit{in vivo}. Taken together, I concluded that the neuronal cell cycle plays an essential role in oligomeric Aβ-induced neuronal cell death, which occurs through CaMKII-ERK1/2 signaling cascade.

Lastly, I investigated the roles of apoptotic proteins in oligomeric Aβ-induced cell death in Chapter 5. I showed that treatment with oligomeric Aβ increased Bim levels but decreased Bcl-2 levels, leading to the activation of Bax and neuronal cell death in hippocampal slice culture and \textit{in vivo}. Furthermore, the inhibition of Bax activity either by Bax inhibiting peptide or \textit{bax} gene knockout significantly prevented oligomeric Aβ-induced neuronal cell death. These findings were the first to demonstrate that Bax plays an essential role in oligomeric Aβ-induced neuronal cell death, and that targeting of Bax may be a therapeutic approach for AD.

In conclusion, signaling pathways of PrP\textsuperscript{C} through cell cycle re-entry are critical in oligomeric Aβ-induced neuronal cell death. Reactivation of the cell cycle in neurons induces DNA damage, leading to apoptotic cell death seen in AD. Current treatments are mainly focused on reducing the production of Aβ and attention needs to be prevention of disease and treatment of molecular and mechanical causes. Targeted therapies directed to inhibiting the binding of Aβ to
PrP\textsuperscript{C} or downstream signaling pathways may provide a broader mechanistic means of protection against AD. Investigating the pathophysiological importance of PrP\textsuperscript{C} and cell cycle re-entry in AD may lead to potential treatment of AD.

**Discussion**

I showed that PrP\textsuperscript{C} is an important receptor for oligomeric Aβ and promotes its neurotoxicity, and that caveolin-1 mediates the neurotoxic signal through PrP\textsuperscript{C}. The signaling pathway leads to cell cycle re-entry, followed by DNA damage accumulation, ultimately leading to apoptosis in neuron.

Lauren *et al.* identified PrP\textsuperscript{C} as a high affinity receptor for oligomeric Aβ and showed that expression of PrP\textsuperscript{C} was essential for oligomeric Aβ-induced synaptic toxicity as determined by loss of long-term potentiation (LTP) [20] and memory impairment in transgenic Alzheimer mice [21]. However, three independent studies failed to confirm a critical role for PrP\textsuperscript{C} *in vivo* and *in vitro* [22-24]. Balducci *et al.* reported that oligomeric Aβ are responsible for cognitive impairment in AD and that PrP\textsuperscript{C} is not required [22]. Another paper suggested that ablation or overexpression of PrP\textsuperscript{C} had no effect on the impairment of hippocampal synaptic plasticity in APPPS1 mice [23]. In the meantime, independent studies, including one from our group, provided additional experimental evidence for the concept that PrP\textsuperscript{C} can act as a mediator of Aβ-induced toxicity. Recent studies confirmed that an anti-PrP antibody targeted to PrP\textsuperscript{C}_{93-102} blocks LTP induced by Aβ-containing AD brain extract [46, 47].
cross-linkage of PrP$^C$ by oligomeric Aβ triggers abnormal activation of cPLA2 and synapse damage [158].

I report here that neuronal cell death induced by oligomeric Aβ was prevented by reducing or eliminating PrP$^C$, or blocking the binding between PrP$^C$ and oligomeric Aβ using either a PrP$^C$ antibody or a decoy PrP$^C$ peptide. My study supports the concept that the PrP$^C$/Aβ interaction is necessary for neuronal cell loss. Actually, recent studies showed additional evidence for the PrP$^C$ can act as a mediator of Aβ-induced toxicity [46, 54, 60]. The expression of PrP$^C$ sensitizes cells to toxic effects of not only Aβ, but also other β-sheet-rich conformers, such as the yeast prion protein Sup35 or designed β-sheet peptides [54].

The molecular neurotoxic mechanisms of oligomeric Aβ through PrP$^C$ remain unclear. NMDA receptor-mediated excitotoxicity might be the downstream mechanism of Aβ neurotoxicity, since our data and others showed that NMDA antagonist blocks the neurotoxicity [59]. Oligomeric Aβ was found to directly or indirectly bind NMDA receptor [17, 18] and PrP$^C$ is also reported to interact with the NR2D subunit, which is a key regulatory subunit of the NMDA receptor [53]. Interestingly, I found Aβ-induced neurotoxicity was significantly reduced by pharmacological blockage of general NMDA and specific NR2B subunit. Consistent with my result, oligomeric Aβ induces early neuronal dysfunction mainly by activation of NR2B-containing NMDA-receptors [80, 159]. Several studies show that NR2A is generally found at the synapse, whereas NR2B is predominantly localized at extrasynaptic sites [85] and enhanced activation of
extrasynaptic NR2B-containing NMDARs is common in AD. Enhanced activation of extrasynaptic NR2B leads to excessive influx of Ca\(^{2+}\) into the cell, which results in inappropriate activation of enzymes (such as calpains and other calcium-regulated enzymes) and mitochondrial dysfunction; this leads to apoptosis [68, 85]. Collectively, these data suggest that abnormal function of NMDA receptor may contribute to the neurotoxicity of oligomeric Aβ through PrP\(^{C}\).

The connection between NR2 subunit and PrP\(^{C}\) are far from being understood. This involves interactions of PrP\(^{C}\) with the protein assemblies and with cellular factors required for signal transmission. I investigated roles of Fyn kinase as a signaling molecule that links two proteins. Fyn is a member of the Src family of tyrosine kinases (SFK) and is a well-known kinase of NR2 subunit. Interestingly, stimulated PrP\(^{C}\) induces Fyn activation in bioaminergic cells [37]. My data shows that oligomeric Aβ induces NR2B phosphorylation through Fyn in a manner dependent on PrP\(^{C}\) and caveolin-1 expression and that caveolin-1 act as a scaffolding protein. Importantly, caveolin-1 knockdown significantly blocked the neurotoxicity. In consistent to my observation, the C-terminal half of the oligomerization domain (i.e. residues 82–101 in Cav-1) binds to and regulates Fyn activity [83]. In addition, caveolin-1 is required for PrP\(^{C}\)-induced Fyn activation [72]. Future studies may further clarify a role of caveolin-1 in PrP\(^{C}\) induced signaling. Taken together, my data suggest that oligomeric Aβ induces cross-link PrP\(^{C}\), leading to activation of Fyn and NR2B through caveolin-1, and neuronal cell death.
Another important issue about PrP\textsuperscript{C}-A\textbeta interaction is to identify binding sites of PrP\textsuperscript{C} to oligomeric A\textbeta for neurotoxic signaling. The pretreatment with the antibody 6D11, which binds PrP\textsuperscript{C}_{93-109}, prevents neuronal cell death by oligomeric A\textbeta, while another anti-PrP\textsuperscript{C} antibody, 6H4, which recognizes PrP\textsuperscript{C}_{144-152}, failed to block oligomeric A\textbeta-induced neuronal toxicity [59]. Furthermore, consistent with the antibody experiments, addition of the peptide corresponding to residues PrP\textsuperscript{C}_{98-107} reduced the neurotoxicity of oligomeric A\textbeta in the hippocampal slice cultures, whereas the peptide corresponding to residues PrP\textsuperscript{C}_{213-230} had no effect on the A\textbeta-induced neurotoxicity. These data indicate it is the essential region PrP\textsuperscript{C}_{98-107} in PrP\textsuperscript{C} that mediates A\textbeta-PrP\textsuperscript{C} interaction. To support this finding, a previous study [20] identified the sequence (amino acids 95-105) as a binding site of PrP\textsuperscript{C} for A\textbeta in a which showed that the treatment with antibody binding this region prevented the interaction and A\textbeta-induced LTP [20] and improved cognitive deficits in aged AD transgenic mice [55]. More recent studies also confirmed that an anti-PrP antibody targeted to PrP\textsuperscript{C}_{93-102} blocks LTP induced by A\textbeta-containing AD brain extract [46, 47]. Furthermore, PrPN, a mutant lacking a large portion of the N-terminal domain, is impaired in both binding to A\textbeta and mediating its toxic effects [54]. Collectively, these findings strongly suggest that PrP\textsuperscript{C}_{98-107} contains the critical amino acid sequence for oligomer A\textbeta-induced synaptic impairment and neuronal cell death. Although increasing number of observation supports the role of PrP\textsuperscript{C} as a receptor for A\textbeta-induced neuronal loss, further research is required to identify the neurotoxic mechanisms through PrP\textsuperscript{C}. 

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Next, I addressed the role of cell cycle re-entry in the neurotoxicity. Neurons, normally in G0 of the cell cycle, as they are terminally differentiated cells, do not undergo cell proliferation. The lines of evidence indicate the upregulation of various cell cycle markers in neurons vulnerable to degeneration in AD, while no evidence exists of actual mitosis. I sought to determine the role of cell cycle re-entry and signaling mechanisms in oligomeric Aβ-induced cell death. I showed here that oligomeric Aβ induces cell cycle re-entry prior to cell death. I also identified that the CaMKII-ERK1/2 signaling pathway, by mediating the degradation of p27Kip, is a key mechanism for neuronal cell cycle re-entry. Importantly, inhibition of ERK/CaMKII signaling pathway and cell cycle re-entry significantly attenuate neuronal cell death induced by oligomeric Aβ. Additionally, genetic inhibition of cell cycle by E2F1 knockout blocks the neuronal cell death. These data suggests that cell cycle-like events in hippocampal slice culture are not a consequence, but a critical upstream mechanism of Aβ-induced cell death. In addition to the active DNA synthesis, I found an accumulation of DNA damage. I believe that the terminally differentiated neurons do not have the proper machinery to deal with the cell re-entering the cell cycle and for DNA synthesis to occur and that the new DNA being synthesized is full of errors. Normally the cell would activate the DNA repair pathway, but when the amount of DNA damage overwhelms the DNA repair system, apoptosis is then being triggered through activation of the ATM/p53 pathway. To support my idea, deficiency of ATM or p53 is more resistant to oligomeric Aβ-induced cell death than their wild-type littermates. DNA repair pathways need to be analyzed to determine when they are
being activated and if the cell downregulates these pathways or if they become overwhelmed. The accumulation of DNA damage and the ability for the cell to keep up with the repair of the DNA may be the limiting factor for initiation of apoptosis.

Apoptosis is regulated by the expression levels of Bcl-2 family proteins. Ku70, a DNA repair factor, can prevent the conformational change by binding with Bax in the cytosol, which leads to the inhibition of Bax-mediated cell death. Previously, a cell permeable Bax-inhibiting peptide (BIP), designed to induce the Bax binding domain of Ku70, was found to rescue cells from Bax-mediated cell death [132-134]. I tested knockdown of Bax and peptides which block the activation of Bax in Aβ-induced cell death. The lack of Bax and BIP application prevents oligomeric Aβ-induced neuronal cell death. Taken all together, it is highly likely that the activation of Bax by the regulation of Bim and Bcl-2 family is crucial for oligomeric Aβ-induced neuronal cell death.

In conclusion, cell cycle re-entry and signaling pathways operating through PrP<sub>C</sub> in neurons are critical in Aβ-induced apoptosis. This is the first study delineating Aβ-induced neurotoxic mechanisms through PrP<sub>C</sub> in neurons. Additionally, I showed that Aβ induced cell cycle re-entry, and the following DNA damage, is an important step in the cell death process. This finding is of great value for developing and testing new therapeutic targets to cure AD.
CITATIONS


