Lack of neuroprotective effects by platelet-derived growth factor against beta-amyloid induced toxicity uncovers a novel hypothesis of Alzheimer's disease pathology

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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ABSTRACT

Aβ oligomer-induced neurotoxicity has become an important area of therapeutic development in treating Alzheimer's disease. Platelet-derived growth factor (PDGF) has been shown to be able to protect neurons against several neuronal insults such as ischemia and HIV1 toxin induced cytotoxicity. These neuroprotective effects correlate well with our previous results that demonstrate the neuroprotective effects of PDGF-BB, one of the PDGF receptor ligand subtypes, against NR2B containing NMDA receptor induced excitotoxicity, a possible underlying cause of Aβ oligomer induced synaptic dysfunction and neuronal death. This project examines the neuroprotective effect of PDGF-BB against $A\beta_{1-42}$ oligomer induced cytotoxicity in both SH-SY5Y cells and primary hippocampal neurons. Cell viability was monitored by MTT assay and the affected signaling pathways were examined using pharmacological methods and Western blotting. The results demonstrated that $A\beta_{1-42}$ oligomer elicited a dose-dependent toxicity with a sign of saturation at higher dosages, PDGF-BB failed to protect neurons against $A\beta_{1-42}$ oligomer induced cytotoxicity. In contrast, $A\beta_{1-42}$ oligomers strongly inhibit PDGF-BB induced mitogenesis in both SH-SY5Y cells and primary neurons. Further investigation using Western blotting to measure PDGF receptor expression and phosphorylation in SH-SY5Y cells showed that $A\beta_{1-42}$ oligomer can inhibit PDGF-BB induced phosphorylation of PDGF β-receptor on Tyr1021, a site that is crucial for PLCγ mediated mitogenesis. These findings not only explained the poor neuroprotective effect elicited by PDGF-BB against $A\beta_{1-42}$ oligomers, but also led to a novel hypothesis that $A\beta_{1-42}$ oligomer may interfere with neurotrophic factor induced neuronal survival, either selectively or perhaps globally. Further exploration on this hypothesis will be able to shed light on this potentially novel mechanism of pathogenesis in Alzheimer's disease.

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DEDICATION

I dedicate this thesis to my husband, George, for being supportive in so many aspects along the journey, to our daughter, Hannah, for her patience and understanding at a very young age, and to my brother, Lei, for being the initial reason why I chose to study life science.

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Chapter I

Introduction

1.1. Alzheimer's disease (AD) and the two pathological hallmarks

Alzheimer's disease (AD) is a progressively developing neurodegenerative disorder of the brain. Over time, patients may lose memories, physical coordination, and eventually die on average within 7 years after the diagnosis[1]. As more baby boomers are reaching age of 65, AD will represent a greater and greater economic and social burden to the society, making the search for efficient treatment, especially at its early stage, an urgent medical research objective.

At the cellular level, the disease is characterized by synaptic dysfunction and neuronal loss. While the etiology of AD remains unknown, senile plaques and neurofibrillary tangles (NFTs) are typically found in the post-mortem brains and are the two well-accepted hallmarks of AD[2]. Senile plaques are primarily extracellular deposits of β-amyloid (Aβ) peptides and NFTs are mainly intracellular deposits of hyperphosphorylated tau, a microtubule-associated protein.

Aβ is produced in all normal brains through a proteolytic process on a membrane-bound protein, amyloid precursor protein (APP). APP can be either cleaved by combination of α- and γ-secretases or β- and γ-secretases. The latter combination leads to the production of Aβ and therefore is classified as the amyloidogenic pathway: Aβ is misfolded and aggregates in β sheets to form senile plaques[3]. There are various forms of Aβ found in senile plaques[4], but it is believed that the longer and more hydrophobic form of Aβ, $A\beta_{1-42}$, is the most toxic and is responsible for the early stage of senile plaque formation[4, 5]. The identification of fibril forms of Aβ led to the initial hypothesis that it was these large precipitates of Aβ that were the pathological cause of AD. However, recent evidence

shows that the much smaller, diffusible Aβ oligomers are equally or more toxic than the larger fibrils and therefore hold more promise as a therapeutic target in reversing the progress of the disease at its early stage[6-9].

Tau exists in 6 isoforms with highly conserved microtubule-binding domains, through which it can stabilize and promote microtubule polymerization in neurons, predominantly in axons[10]. Tau is normally phosphorylated and soluble but in tauopathies, tau-related pathogenesis including that in AD, it becomes hyperphosphorylated and can accumulate into paired helical filaments, the major component of NFTs[10, 11]. In AD, about 45 phosphorylation sites of tau have been determined, which makes the corresponding protein kinases the possible therapeutic targets[10].

Interestingly, recent studies have linked the tauopathy to Aβ toxicity. De Felice and colleagues found that tau hyperphosphorylation can be induced by Aβ oligomers[12]. A few protein kinases that can phosphorylate tau can be also activated in Aβ induced signaling[13, 14]. Other studies show that Aβ induced neurotoxicity can be tau-dependent[15, 16].

Current lines of investigation into the therapeutic treatment of AD are focused on finding ways to prevent and reverse the brain damage caused by Aβ and tau. One of the approaches is to use neuroprotective agents, such as neurotrophic factors, to prevent further neuronal loss and promote neurite growth in the early stages of AD.

1.2. PDGF neuroprotection

PDGF (platelet-derived growth factor) is a neurotrophic factor that has been demonstrated to be neuroprotective following ischemic events[17, 18], oxidative stresses[19, 20] and insults from HIV-1 proteins, the putative cause of HIV-associated dementia[21-23].

The PDGF ligands are hetero- or homo-dimers of PDGF-A, B, C or D chains[24]. PDGF ligands bind to PDGF receptors, either α- or βreceptor isoforms, with different affinities[25-27]. PDGF-A chain and PDGF-B chain, as well as PDGF receptors are widely expressed in both developing and mature central nervous system (CNS)[28]. PDGF-BB is the major species that has been implicated in neuroprotective effects.

One of the possible mechanisms of PDGF neuroprotection is through inhibition on N-methyl-D-aspartate (NMDA) receptors. This inhibition has been reported in a number of independent studies[29-31], including ours[32, 33]. In one of our studies, we identified Abelson tyrosine kinase (Abl or c-Abl to differentiate with Arg, Abl related gene, which belongs to the same family of Abl) as a downstream effector of PDGF that inhibits NMDA currents by modifying the actin cytoskeleton[32].

1.3. NMDA receptor and AD

NMDA receptor plays an essential role in synaptic plasticity and memory[34] as well as excitotoxicity[35]. NMDA receptors are ion channels that are both ligand-gated and voltage-dependent: depolarization of membrane relieves the basal channel blockade by Mg^{2+} and when its natural ligand, glutamate, and co-ligand, glycine, bind, the channel opens and is permeable to Na^{+}, K^{+} and Ca^{2+} [36]. Ca^{2+} influx through NMDA receptor into postsynaptic neurons triggers multiple biochemical cascades that lead to long term potentiation (LTP), a mechanism that underlies learning and memory[37]. However, overload of intracellular Ca^{2+} caused by overstimulation by glutamate on NMDA receptors will lead to excitotoxicity to neurons[35].

The NMDA receptor is a tetramer containing two NR1 and two NR2 subunits, and NR2 can be NR2A, 2B, 2C or 2D subtype[34]. NR2A-containing NMDA receptors are mainly expressed at synaptic sites while NR2B-containing NMDA receptor can be found both synaptically and extrasynaptically[38]. Signaling through NR2A-containing NMDA receptors results in neuroprotective effects while NR2B-

containing NMDA receptor signaling is responsible for triggering cell death following excitotoxic insults[39].

Recently, accumulating evidence shows NMDA receptors play important roles in AD pathology. NMDA receptors, especially NR2B-containing NMDA receptors, are required for Aβ oligomer induced neurotoxicity and synaptic dysfunction[40-42]. These findings are well correlated with the clinical effects of memantine, a non-competitive NMDA receptor antagonist approved by FDA in 2003 in treating moderate to severe AD[43].

1.4. c-Abl and AD

c-Abl is a non-receptor tyrosine kinase that is well known in oncogenesis as part of Bcr-Abl fusion protein resulted from Philadelphia chromosomal translocation (9:22) in chronic myeloid leukemia (CML), where the strictly regulated tyrosine kinase in its normal form becomes constitutively activated within the fusion protein[44]. STI571, also known as imatinib or Gleevec®, is a small molecule inhibitor of c-Abl that has been successfully applied in the treatment of CML[45].

c-Abl can be found at several subcellular locations including plasma membrane, cytoskeleton and nucleus[46]. It has been implicated in various biological functions from promoting cell growth to mediating cell death[47]. Several membrane receptors, including PDGF receptors, signal through c-Abl[48]. Plattner and colleagues have shown that PDGF receptor can activate c-Abl in a Src and PLCγ-1 dependent manner[49-51]. c-Abl has actin-binding domains that can directly interact with F-actin and G-actin in the cytoskeleton[47]. It can also interact with several protein families to regulate actin dynamic indirectly. One such family is Rho family of small GTPases. Jones and colleagues found that Abl promote dendrite outgrowth through a RhoA/ROCK dependent manner[52]. c-Abl is expressed in both presynaptic and postsynaptic neurons and is concentrated in synapse-rich region, which correlates with its functions in maintaining synapse morphology and efficient neurotransmitter

release[48]. On the other hand, c-Abl is also implicated in inducing apoptosis responding to DNA damage and oxidative stress[53]. Numerous studies suggest c-Abl interacts with p73, a member of the p53 family of tumor-suppressor proteins, to induce apoptosis in response to DNA damage in nucleus[54-57]. In the cytoplasm, c-Abl is activated by oxidative stress and was implicated in mediating H_2O_2 induced apoptosis[58], where its F-actin binding domain (FABD) is required[59].

A few studies have shown possible roles of Abl (both c-Abl and Arg) in AD pathology. One such role is that c-Abl can be a mediator of Aβ induced apoptosis. Alvarez and colleagues found that Aβ fibril can activate c-Abl and can mediate Aβ induced apoptosis[60]. Another role of Abl in AD pathology was attributed to its ability to phosphorylate tau. Abl can phosphorylate tau at Tyr197 (by Arg)[61] and Tyr394 (by Arg and c-Abl)[61, 62], which are among the phosphorylated sites found in the hyperphosphorylated tau in NFTs[10]. In both categories, STI 571, the inhibitor of Abl, was shown to be able to reverse the pathological effects mediated by Abl both *in vitro* and *in vivo*[63, 64].

1.5. Hypothesis, methodology and project outline

Based on previous studies and the knowledge gained from investigations into the roles of NMDA receptors and c-Abl in AD pathology, we hypothesized that PDGF might be able to protect neurons against Aβ oligomer induced neurotoxicity through inhibition on NR2B-containing NMDA receptors, and that c-Abl may mediate this neuroprotection.

The MTT assay is a well-accepted colorimetric method in measuring Aβ toxicity in various cell lines and primary neuronal cultures. The assay is based on the ability of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide) into purple formazan crystals, and the amount of formazan production correlates with the viability of cells[65]. The assay is relatively fast and easy to perform compared to other methods in Aβ toxicity studies and therefore was chosen to investigate PDGF neuroprotection against Aβ oligomer toxicity in both SH-SY5Y cells and primary neurons, as

well as the involvement of NR2B-containing NMDA receptor and c-Abl with pharmacological methods.

Western blotting is a well-established analytical technique to quantify protein levels in a given sample of tissue homogenate or extract[66]. Proteins are separated in gel electrophoresis and transferred onto a membrane that can be probed using antibodies that specifically recognize the protein of interest. Advances in antibody development used in Western blotting have allowed researchers to be able to not only evaluate the level of certain protein in total but also quantify the phosphorylation of the protein at specific phosphorylation sites.

The project was organized into three parts:

1. Optimization of Aβ oligomer preparation and MTT assay in SH-SY5Y cells.

The role of Aβ oligomers in AD pathology was only recognized recently. Aβ oligomers act quite differently from Aβ fibrils and monomers, thus it is very important to make sure the preparation of the oligomers is done properly and consistently. Aβ is a protein that will aggregate very easily, if the conditions are not well-controlled, fibrillar Aβ may ultimately become the dominant form. In literature, overnight incubation of Aβ in cell medium at low temperature, such as 4°C, has been proven to be able to produce stable, reproducible oligomers[67]. However, the details of preparation vary among studies depending on the cells employed. Therefore, an optimizing step was taken, specifically for our cell line, to ensure the prepared oligomers can exert a significant and reproducible toxic effect.

The MTT assay has been established as a valuable tool to measure cell viability and proliferation for many years while it constantly evolves ever since. In literature, one can easily find multiple modified protocols being applied even for very similar research. This made it very hard to decide which one to

follow. Therefore, steps were taken at the beginning of the project to optimize the procedures involved in MTT assay in order to achieve reproducible and reliable results for the study.

2. PDGF neuroprotection against Aβ oligomer toxicity in SH-SY5Y cells and primary neurons, including the examination of the roles of NR2B-containing NMDA receptor and c-Abl.

Once the best protocols for Aβ oligomer preparation and MTT assay were determined, Aβ toxicity and PDGF neuroprotective effect were examined in SH-SY5Y cells and confirmed in primary neurons. Various dose-response experiments with specific time variations were performed. To test the role of NR2B-containing NMDA receptor and c-Abl in this proposed pathway, an antagonist of the specific NMDA receptor subtype and an inhibitor of c-Abl were used.

3. Further mechanism exploration using Western blotting.

At the end of the project, possible mechanisms of the observed effect of Aβ and PDGF in SH-SY5Y cells were further explored using Western blotting. SH-SY5Y cells were treated with the same dosages of $A\beta_{1-42}$ oligomer and PDGF-BB as those used in MTT assay and lysed for Western blotting analysis. Interesting results were obtained from the analysis that explained the unexpected results in the cell viability assays and may provide the foundation of a novel hypothesis on Aβ induced neurotoxicity in AD.

Chapter II

Results and Discussions

2.1. Optimization of Aβ oligomer preparation and MTT assay

The usages of Aβ and MTT assay were new to the laboratory. Therefore, a significant amount of time was spent on optimizing both the assay and the preparation of $A\beta_{1-42}$ oligomer at the beginning of this project.

2.1.1. Working with $A\beta_{1-42}$: choosing the right product and establishing the aliquoting protocols

One of the major challenges was how to properly work with the commercially available $A\beta_{1-42}$. This includes the selection of proper $A\beta_{1-42}$ products as well as the establishment of aliquoting protocols that involves choices of chemicals and equipment, considerations on the amount to be aliquoted and the storage methods.

The $A\beta_{1-42}$ used in this project was supplied by rPeptide (Georgia, USA). The DNA sequence encodes the human beta-amyloid (1-42) sequence and is expressed in *E.coli*. The supplier provides two kinds of $A\beta_{1-42}$. One kind is associated with counter ions, which are obtained in the protein purification process to protonate the basic amino acid residues and N-termini. Several $A\beta_{1-42}$ products with counter ions from NaOH, TFA (trifluoroacetate, $CF3COO^-$), or HCl are available. Another kind of Aβ product is HFIP (hexafluoroisopropanol)-treated. HFIP is an organic solvent that is capable of solubilizing peptides and eliminating any pre-existing secondary structure, such as β-sheets[67]. Since my project was focused on Aβ oligomers, but not other forms of Aβ aggregates, such as profibril or fibril, I needed to eliminate any pre-existing structures from the starting material to ensure there was

no other forms of aggregates that might develop during the preparation process. Therefore, HFIP-treated $A\beta_{1-42}$ was chosen.

The aliquoting step was taken for several reasons: 1) the commercially available $A\beta_{1-42}$ is quite expensive; 2) the nature of the experiments doesn't require a large amount of $A\beta_{1-42}$ for each trial; 3) $A\beta_{1-42}$ in solution is not stable and $A\beta_{1-42}$ oligomer has to be prepared fresh, 24hr before use.

The appropriate chemicals and equipment were purchased and prepared based on Stine's protocol[67] with some modifications. Since HFIP-treated $A\beta_{1-42}$ had been chosen, the solvent that was used to re-suspend the lyophilized peptide in the first aliquoting step was HFIP. Since moisture can cause peptide degradation[68], the last step was performed in a vacuum desiccator and $A\beta_{1-42}$ aliquots were stored over desiccant in a glass desiccator at -20°C. Amber microcentrifuge tubes were used to store the aliquots to reduce the effects of light, another source of protein degradation. This protocol allows for stable peptide storage for several months to years[67].

The amount for each $A\beta_{1-42}$ aliquot was calculated based on the final application in 96-well plates for MTT assay and 6-well plates for Western blotting. Although the exact amount of $A\beta_{1-42}$ varied from experiment to experiment due to the different conditions being tested, a proper unit amount of $A\beta_{1-42}$ was determined for both MTT assay and Western blotting to eliminate any waste. Therefore, multiple aliquots were used for each experiment depending on the specific conditions involved. A slight addition (about 5%) to the desired amount was applied in the calculation since there were hundreds of pipetting steps involved in each experiment that could lead to a significant build-up of small pipetting errors. The concentration of HFIP solution in the re-suspension step was adjusted to a lower concentration (1 mg/mL) instead of 1 mM in Stine's original protocol, for easier measurement and calculation.

Each $A\beta_{1-42}$ aliquot was labeled with a number in the order of the addition of the HFIP-dissolved $A\beta_{1-42}$ stock from each vial. The reason for doing this was based on the practice of always getting fewer aliquots than expected. For example, according to calculation, I was supposed to get 17.7 aliquots from 1 mg of $A\beta_{1-42}$ with 56.46 μg in each aliquot. However, in reality there might just be 16.2 aliquots. This phenomenon was due to the nature of HFIP being highly volatile. During the process of aliquoting under the fume hood, HFIP kept evaporating when each aliquot was prepared with the addition of the same volume of the solution. As a result, the aliquots being prepared at the end contained more $A\beta_{1-42}$ than the aliquot being prepared at the beginning. This problem could not be avoided even the process was done quickly. The different amount between the first aliquot and the last aliquot led to about 10% difference on the concentrations of the $A\beta_{1-42}$ oligomer stock solution (100 μM) prepared on day 2 (see Table 1). The labeled number was used to track the order of the preparation so a combination of aliquots that were prepared earlier and later could be used to help maintain consistency among experiments.

2.1.2. MTT assay: the four-day routine

The major portion of the project was to test PDGF-BB neuroprotection against $A\beta_{1-42}$ oligomer induced toxicity using MTT assay. Every trial followed the same four-day routine (Table 1) to generate results and all the key aspects in this routine were carefully considered and/or optimized.

Table 1: Four-day routine of MTT assay in the study of PDGF-BB neuroprotection against $A\beta_{1-42}$ oligomer induced toxicity.

Day 1	Plating cells
Day 2	Serum starvation and $A\beta_{1-42}$ oligomer preparation
Day3	Treatment
Day4	MTT assay - adding MTT, MTT formazan solubilization and absorbance measurement

Day 1: Determination of the best cell densities

A good resolution for MTT assays depends on identifying an ideal working cell density because the MTT assay measures the activity of metabolically viable cells. If there are too many cells, the toxic effect may not be detectable by the assay since only a small portion of cells will be affected.

According to the manufacturer's instructions, the recommended cell density for the MTT (Sigma) assay was not more than $10^6/cm^2$. However, cell densities lower than 10^5/ml ($3.1\times10^4/cm^2$, converted according to the known surface area of each well to be $0.32cm^2$ and volume of cell culture in each well to be 100µl in the 96-well plates being used) were common in literature. There was about a $10^2/cm^2$ difference between the recommended maximum density and those in literature.

In order to find the best cell density for $A\beta_{1-42}$ oligomer toxicity in SH-SY5Y cells, I started with two cell densities, 10^5/ml ($3.1\times10^4/cm^2$) and 10^6/ml ($3.1\times10^5/cm^2$). As can be seen in Figure 1, the lower cell density provided a better resolution toward the same dosage of $A\beta_{1-42}$ oligomer. Therefore the working cell density was determined in the 10^5/ml range.

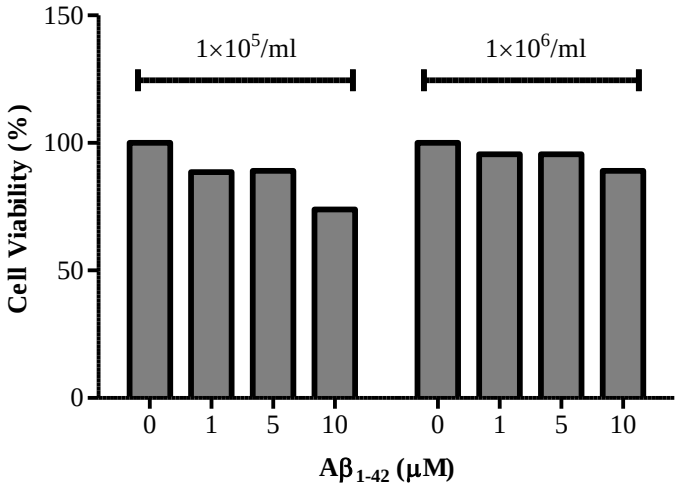


Figure 1: Determination of cell densities for MTT assay. SH-SY5Y cells were plated in two densities in 96-well plates, 1×10^5/ml and 1×10^6/ml, and exposed to $A\beta_{1-42}$ oligomer at various concentrations. Cell viability was measured by MTT assay (n=1).

Within this determined range, several densities were tested along with other optimization steps to narrow down the best working density (Figure 2). I found 0.5×10^5/ml and 1×10^5/ml provided the most consistent results and working with either of these two densities did not make any difference on $A\beta_{1-42}$ oligomer induced toxicity in SH-SY5Y cells. However, 1×10^5/ml was mostly used as it always gave clear color differences among treatments that were readily observable.

Since primary neuronal cells are more challenging to grow compared to SH-SY5Y cells and we had not previously grown primary neurons on 96-well plates, I plated primary neurons on 96-well plates in two densities, 0.5×10^5/ml and 1×10^5/ml. The results showed no difference with respect to the $A\beta_{1-42}$ oligomer induced toxicity in the two cell densities in primary neurons (Figure 3 and Figure 4).

Day 2: Serum starvation and $A\beta_{1-42}$ oligomer preparation

Prior to beginning the assay, the cells were serum-starved. The serum starvation step was conducted for two reasons. The first reason was to reduce cell division promoted by serum components and encourage the development of neuronal features, a process called differentiation. The second reason was to remove PDGF specifically as it is present in the serum supplement.

Cells were serum starved about 24hr after being plated. This 24hr time period was given to allow the cells to establish attachment after plating. After the medium was changed to serum-free medium in the serum starvation step, another 24hr was given to encourage the cells to develop neuronal features.

Another key benefit of serum starvation was to remove phenol red from the culture. Phenol red is a pH indicator that is commonly added to cell culture medium to monitor the pH changes that are crucial for cell growth. However, the presence of phenol red would contribute to a higher background absorbance and decrease the sensitivity of MTT assay. Therefore, I used phenol red free medium in

the serum starvation step instead of regular serum-free medium. This removal of phenol red from the medium helped eliminate the background noise and enhanced the sensitivity of the measurement.

$A\beta_{1-42}$ oligomer preparation was performed on day 2 as well because there was a 24hr-incubation at 4°C required for oligomer formation immediately before the treatment.

In the literature, two $A\beta_{1-42}$ oligomer preparation protocols were commonly used. One was determined by Stine and colleagues[67] and another was developed by Lambert and colleagues[6]. Both protocols produced high quality oligomers evaluated by atomic force microscopy (AFM) and Western blotting[6, 67]. The major similarities and differences between the two protocols in the order of the procedures are summarized in Table 2.

Table 2: Similarities and differences between Stine's and Lambert's protocols.

	Stine's	**Lambert's**
Dissolved in DMSO (5mM) with sonication (10min)	Yes	No
Diluted in cold medium (100μM) with vortex	Yes	Yes
Incubated at 4°C, 24hr	Yes	Yes
Centrifuged before treatment	No	Yes

As can be seen in Figure 2, Stine's protocol gave a better correlation between the cell viability signal and the known cell density compared to Lambert's protocol. The usage of DMSO in dissolving the HFIP treated $A\beta_{1-42}$ film as the first step in Stine's protocol may play an essential role in the better result. This step was suggested to be indispensable to ensure fully solubilization of the HFIP treated peptide and proven to be able to produce uniform and unaggregrated oligomer structures which could not be generated by directly dissolving in water[67]. Additionally, the centrifugation step did not seem to be necessary for the small amount of $A\beta_{1-42}$ in each aliquot in this project since there was no visible pellet formed after the step. Therefore Stine's protocol was chosen for the following viability assays.

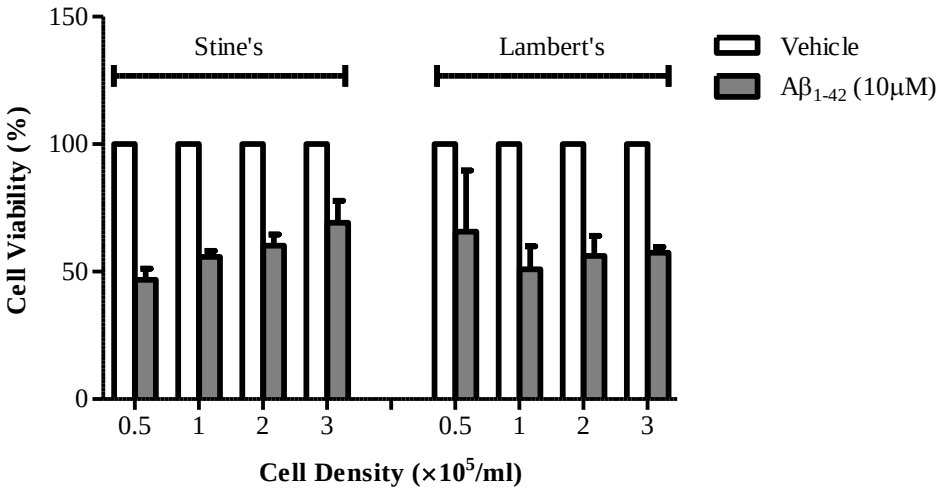


Figure 2: Determination of $A\beta_{1-42}$ oligomer preparation protocols. SH-SY5Y cells in different cell densities were exposed to 10 μM $A\beta_{1-42}$ oligomer for 24hr. Two $A\beta_{1-42}$ oligomer preparation protocols, Stine's protocol and Lambert's protocol, were examined. Cell viability was measured by MTT assay (n=3-4).

Day 3: Treatment

Cell treatment was performed after $A\beta_{1-42}$ oligomer preparation was completed. Reagents were added sequentially without mixing even for the co-treatment to eliminate any possible interactions between the reagents prior to the exposures to the cells. For $A\beta_{1-42}$ oligomer, once the incubation time was completed, the aliquots were combined as one pool to eliminate errors caused by any discrepancy that may exist between the aliquots. Since MTT assay directly measures absorbance, which is proportional to the concentration according to Beer-Lambert Law, the volume in each well was maintained the same for all treatments. Therefore, each well was treated with the same volume in each treatment, and for the control wells where the reagent was absent, the solvent of that reagent was added to ensure the same volume per well. For $A\beta_{1-42}$ treatment, the additional amount of solvent was prepared and incubated in the same manner as for the reagent on day 2.

Day 4: Optimization of MTT formazan solubilization

After 3hr of incubation with MTT, MTT formazan appeared as needle-like purple crystals around the cells. These crystals have to be dissolved into solution in a solubilization step before the absorbance is measured. This solubilization step is very important because it will cause errors in the absorbance reading if the crystals are not completely dissolved. While many solubilization buffer recipes exist, I chose the one used in Sigma's TOX1 MTT kit to match well with the MTT that was also purchased from Sigma. Beside the solubilization recipes, the solubilization methods were also quite different among different study groups. One might take off the medium before adding solubilization buffer while the other would leave the medium in and incubate with the solubilization buffer overnight. To determine the best method, I tried several options (Table 3) and generated a standard curve against cell densities for each of them (data not shown). Based on the standard curves, Option 1 was chosen because it showed a better sensitivity at the desired cell density range and was relatively less labor and time intensive.

Table 3: Optimization of MTT formazan solubilization.

Option 1	Medium-in, 3hr incubation
Option 2	Medium-in, overnight incubation
Option 3	Medium-off , 3hr incubation
Option 4	Medium-off , overnight incubation

2.2. PDGF neuroprotection against Aβ oligomer toxicity

2.2.1. $A\beta_{1-42}$ oligomer toxicity

Aβ toxicity has been studied in many ways in different cell types. Although SH-SY5Y cells are one of the common cell lines used in these studies, there was no clearly demonstrated dose-response curve for Aβ toxicity in SH-SY5Y cells previously in literature. In this project, SH-SY5Y cells were exposed to 10 nM to 10 μM $A\beta_{1-42}$ oligomer for 24hr and a dose response curve was generated (Figure 3). The resulting curve showed a concentration-dependent decrease in cell viability in response to $A\beta_{1-42}$ oligomer treatment in SH-SY5Y cells.

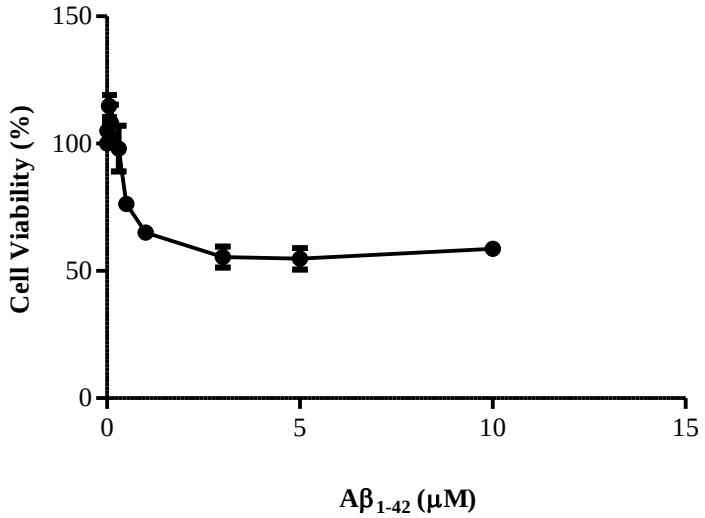


Figure 3: $A\beta_{1-42}$ oligomer induced toxicity in SH-SY5Y cells. SH-SY5Y cells ($0.5\text{-}1\times10^5$/ml) were exposed to different concentrations of $A\beta_{1-42}$ oligomer from 10 nM-10 μM for 24hr and the cell viability was measured by MTT assay (n=4).

A series of dose-response curves was also obtained from primary hippocampal neurons. These dose-response curves were in a similar shape as observed for SH-SY5Y cells. However, the primary neurons seemed to be more susceptible to the same amount of $A\beta_{1-42}$ oligomer than SH-SY5Y cells. This is common for other toxicity effects as well and probably due to the nature of primary cells

being more vulnerable under culturing condition[69], or possibly because they are "true neurons" that lack the ability to divide. Another characteristic of these curves is that the curves for DIV (days *in vitro*) 5 neurons and DIV10 neurons are largely similar. This indicates that the development of neuronal network within 10 days does not have significant impact on the toxic effect.

The dose-response curve obtained here will provide valuable information for similar studies on other neuroprotective agents against $A\beta_{1-42}$ oligomer in SH-SY5Y cells and primary neurons in the future.

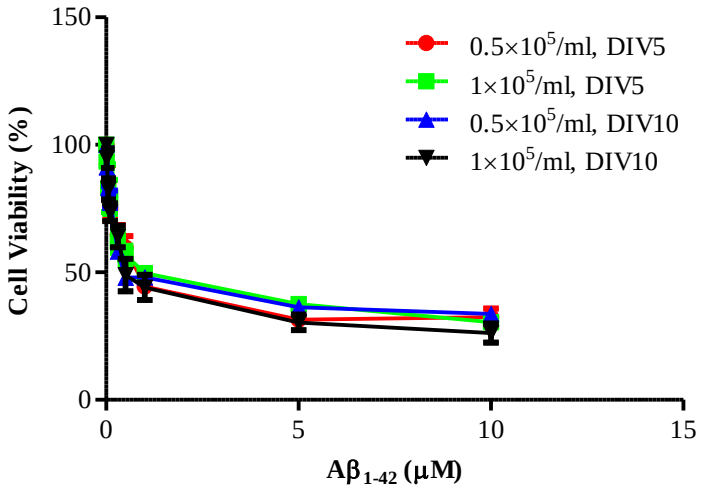


Figure 4: $A\beta_{1-42}$ oligomer induced toxicity in primary hippocampal neurons. Embryonic mouse hippocampal neurons (0.5 and 1×10^5/ml) were exposed to different concentrations of $A\beta_{1-42}$ oligomer from 10 nM-10 μM for 24hr and the cell viability was measured by MTT assay (n=4-5).

In the dose-response curves for both SH-SY5Y cells and primary neurons, there was an interesting feature: a sign of saturation of $A\beta_{1-42}$ oligomer induced toxicity. The relative cell death was more sensitive at lower dosages of $A\beta_{1-42}$ oligomer while at concentration higher than 1 μM there was no significant further reduction of cell viability.

This sign of saturation is very similar to what one would expect to see in a typical ligand-receptor binding curve where a saturation of binding will be reached at certain concentration of the ligand.

Therefore, it is possible that $A\beta_{1-42}$ oligomer may bind to membrane receptors to elicit its toxicity. This possible binding mechanism is consistent with findings from other studies. For example, Klein's group found that Aβ oligomer can bind to two membrane proteins from rat hippocampus and cortex with high binding affinity (~1 nM range)[70].

2.2.2. Other methods of measuring $A\beta_{1-42}$ oligomer toxicity

The MTT assay has been well accepted as a simple yet valuable method to measure Aβ (both fibril and oligomer) toxicity. However, as with other methods, it has its own limitations: it only reflects the ability of cells to reduce MTT and possibly the ability to transport the MTT formazan out of cell membrane by exocytosis[71-73]. Although the impaired metabolic activity and vesicle trafficking might be originated from the same trigger that causes other parallel toxic effects, it would be worthwhile to use other assays to confirm the MTT reduction results in the future when it comes to a more advanced stage of screening for neuroprotective agents against Aβ toxicity. In this way, one can look into if the neuroprotective agents can protect neurons against other toxic effects caused by Aβ. There are several other simple methods that are commonly used in measuring various aspects of Aβ toxicity (Table 4).

Table 4: Commonly used assays in Aβ toxicity studies.

Assays	Measuring or Identifying	Indicating
LDH assay	Lactate dehydrogenase release	Damaged membrane
ATP assay	Cellular ATP level	ATP depletion
TUNEL assay	DNA fragmentation	Apoptosis

The LDH assay is used to measure membrane integrity[74]. Lactate dehydrogenase (LDH) is released when cell membrane is damaged. The assay measures LDH activity by introducing a coupled colorimetric reaction. The increase of LDH activity correlates to the increase of lysed cells. Compared to the MTT assay that measures the changes in metabolic activities happening in the early

stage of Aβ induced cell-death, LDH assay measures the later stage of the process that involves cell membrane rupture.

Another method is to measure cellular ATP levels using a bioluminescent method where an enzyme, luciferase, is used to catalyze the formation of light from ATP and luciferin and the light intensity is linear to ATP concentration[75]. ATP depletion has been considered as a sign of Aβ induced neuronal degeneration that involves mitochondrial dysfunction that leads to synaptic loss and neuronal death[76-78].

TUNEL (TdT-mediated dUTP Nick End Labeling) assay is a qualitative method to detect DNA fragmentation, a key indication of apoptosis[79]. TdT stands for terminal deoxynucleotidyl transferase, an enzyme that catalyzes the addition of fluorochrome-tagged dUTPs to the nicks that contains free 3′-OH termini in fragmented DNA. This method has been widely used in studies where apoptosis might be involved, including Aβ toxicity[80, 81] and PDGF neuroprotection[17, 23].

Besides these simple methods, one can also monitor the change of neuron-specific protein, MAP2 (microtubule-associated protein 2) in primary neurons in Aβ toxicity studies. We have previously used this method in studying PDGF neuroprotection against NMDA induced toxicity in primary neurons[33]. It has been shown that the loss of MAP2 immunoreactivity is proportional to neuronal cell loss[82].

2.2.3. PDGF-BB neuroprotection against $A\beta_{1-42}$ oligomer toxicity and the involvement of NR2B containing NMDA receptors

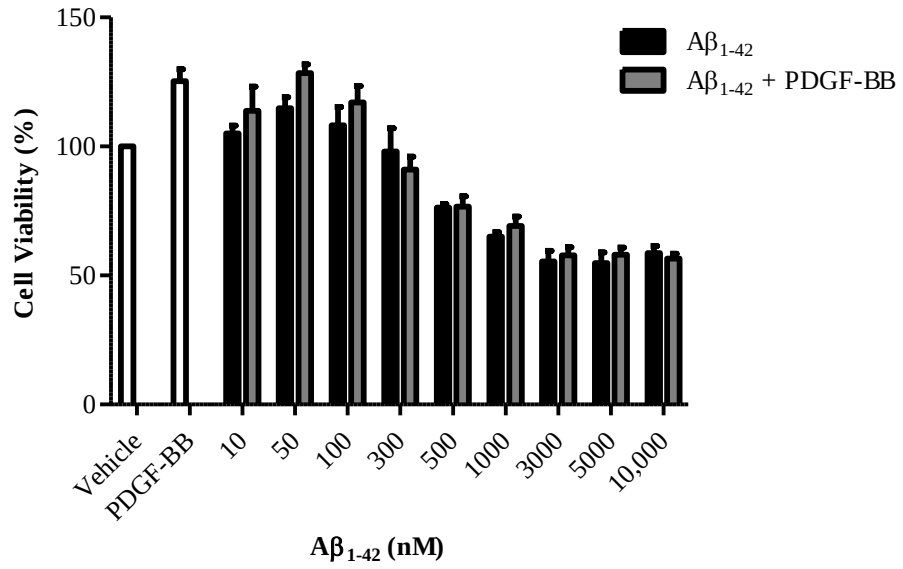
PDGF-BB neuroprotection against $A\beta_{1-42}$ oligomer was examined in several experimental protocols where both PDGF-BB and $A\beta_{1-42}$ concentrations were varied and PDGF-BB was also given additional 30min before $A\beta_{1-42}$ treatment in SH-SY5Y cells. Table 5 is a summary on how the experiments were presented in the corresponding figures for SH-SY5Y cells.

Table 5: Examination of PDGF-BB neuroprotection against $A\beta_{1-42}$ oligomer toxicity in SH-SY5Y cells.

	$A\beta_{1-42}$ dose-response, PDGF-BB=10 ng/ml	**PDGF-BB dose-response, $A\beta_{1-42}$=5 µM**
Co-treatment	Figure 5	Figure 7
PDGF-BB 30min pretreatment before $A\beta_{1-42}$ exposure	Figure 6	Figure 8

Although PDGF-BB has been implicated to be neuroprotective against many neurodegenerative agents, PDGF-BB failed to provide a strong protection against $A\beta_{1-42}$ oligomer induced toxicity in both SH-SY5Y cells (Figures 5-8) and primary neurons (Figures 9-10): at all the tested concentrations for both $A\beta_{1-42}$ and PDGF-BB, PDGF-BB elicited a slight to no neuroprotective effects, even when PDGF-BB was applied 30min prior to induce the neuroprotective cascades before the exposure to $A\beta_{1-42}$ oligomer (Figures 6 & 8). Clearly, PDGF-BB-induced neuroprotective effects were not sufficient enough to protect neurons against $A\beta_{1-42}$ oligomer induced toxicity.

A simple question arose at this point: was PDGF-BB given enough time to elicit its protective effect against $A\beta_{1-42}$ oligomer? Previous evidence shows that PDGF-BB is able to inhibit NMDA-evoked current in a manner that depends on NR2B containing NMDA receptors and reduce the surface expression of this NMDA receptor subtype within 10min, possibly through internalization of the receptors[33]. Since the mechanism of PDGF neuroprotection against Aβ oligomer toxicity was hypothesized to be through inhibition on NR2B containing NMDA receptors, 30min pre-treatment should have been able to prevent the damage caused by $A\beta_{1-42}$ through these receptors. However, this was not the case. One explanation could be the NMDA receptors are not involved in the proposed pathway. Further experiments on the involvement of NR2B containing NMDA receptor corroborated this explanation. As seen in Figure 11, application of Ro25-6981, a selective antagonist of NR2B containing NMDA receptors, did not alter $A\beta_{1-42}$ oligomer induced toxicity any further and the slight neuroprotective effect was unchanged.



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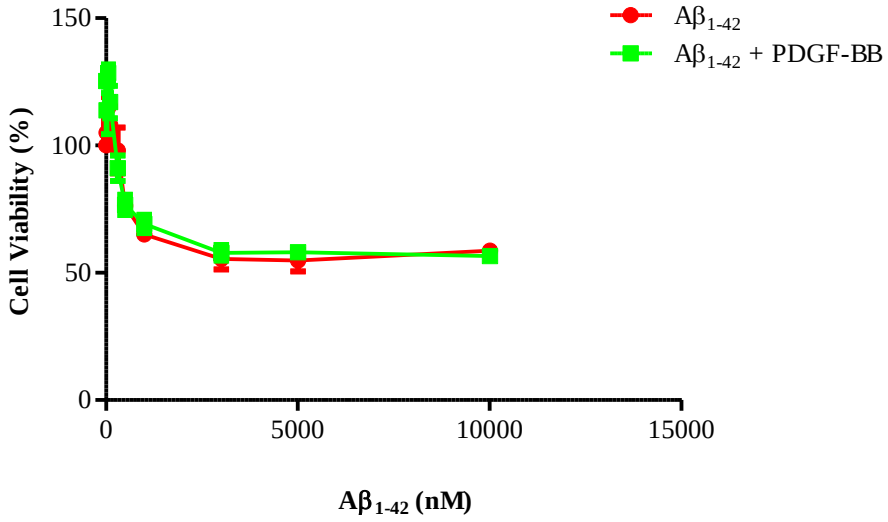
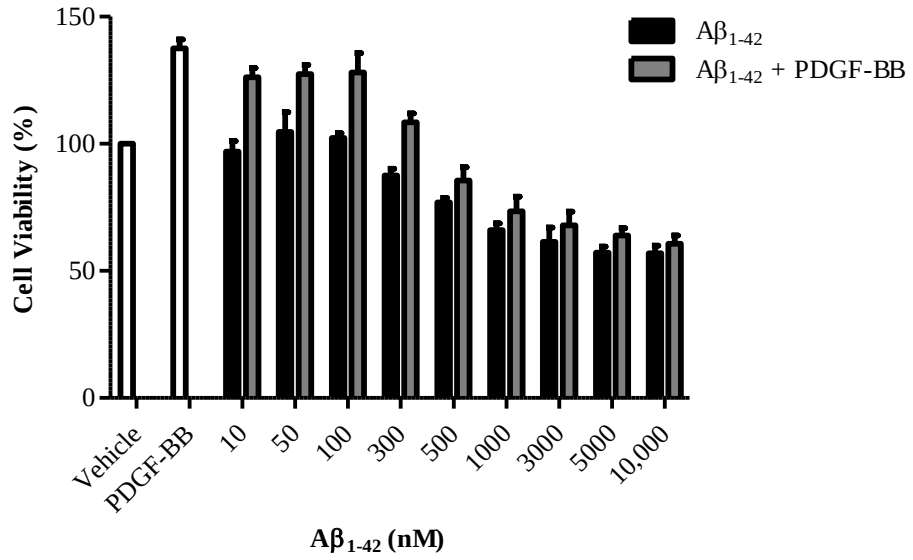


(B)

Figure 5: Neuroprotective effect of PDGF-BB at 10 ng/ml against toxicity of $A\beta_{1-42}$ oligomer at various concentrations (co-treatment) in SH-SY5Y cells. (A) (B) SH-SY5Y cells ($0.5\text{-}1\times10^5$/ml) were exposed to $A\beta_{1-42}$ oligomer at various concentrations in the presence or absence of PDGF-BB at 10 ng/ml for 24hr. Cell viability was measured by MTT assay (n=4).



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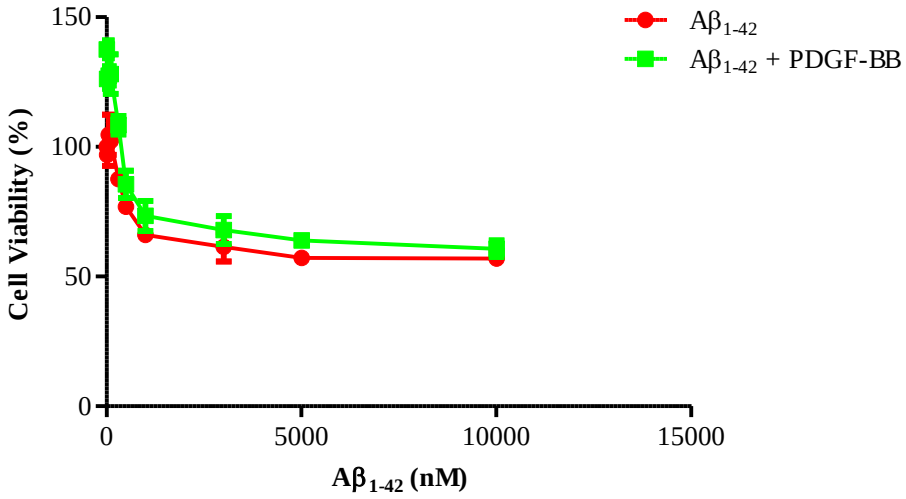
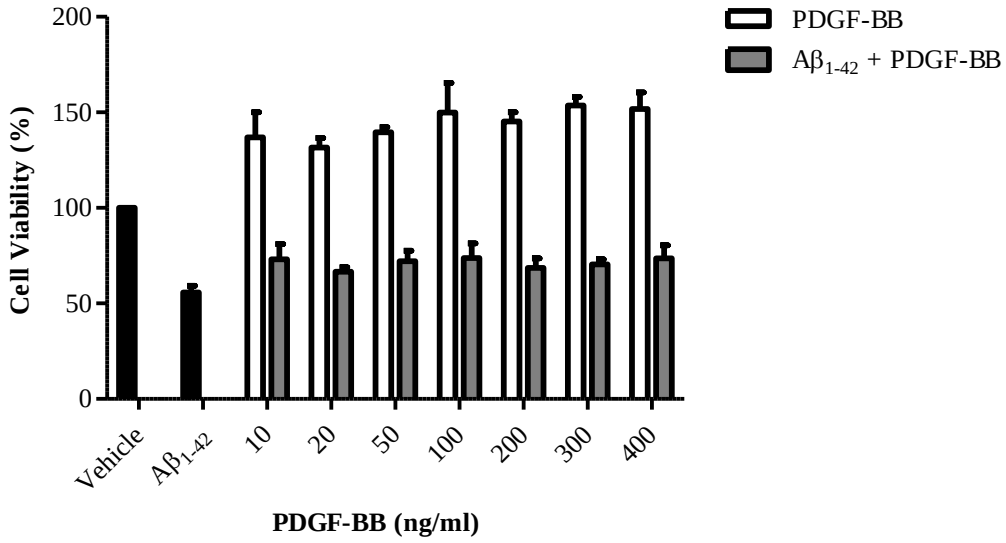
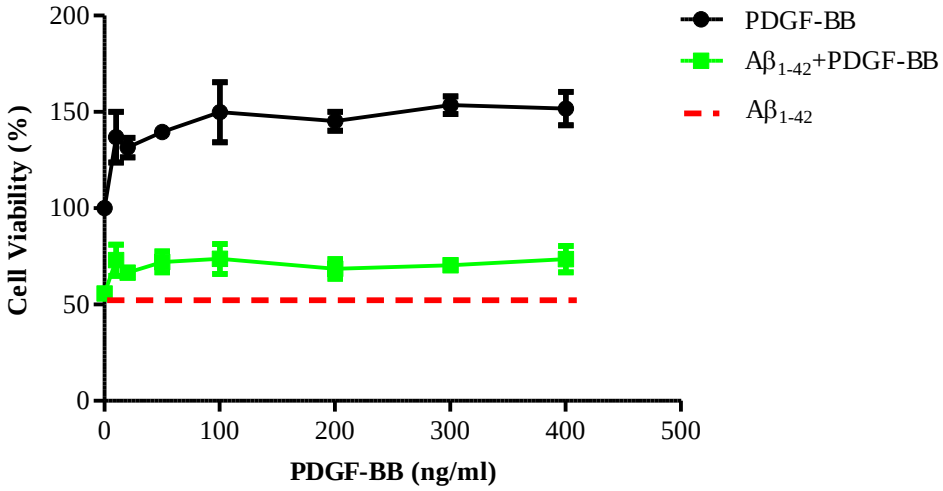


(B)

Figure 6: Neuroprotective effect of PDGF-BB at 10 ng/ml against toxicity of $A\beta_{1-42}$ oligomer at various concentrations (PDGF-BB 30min pretreatment) in SH-SY5Y cells. (A) (B) SH-SY5Y cells (1×10^5/ml) were exposed to $A\beta_{1-42}$ oligomer at various concentrations for 24hr in the presence or absence of PDGF-BB at 10 ng/ml that was added 30min before the exposure to $A\beta_{1-42}$. Cell viability was measured by MTT assay (n=6).

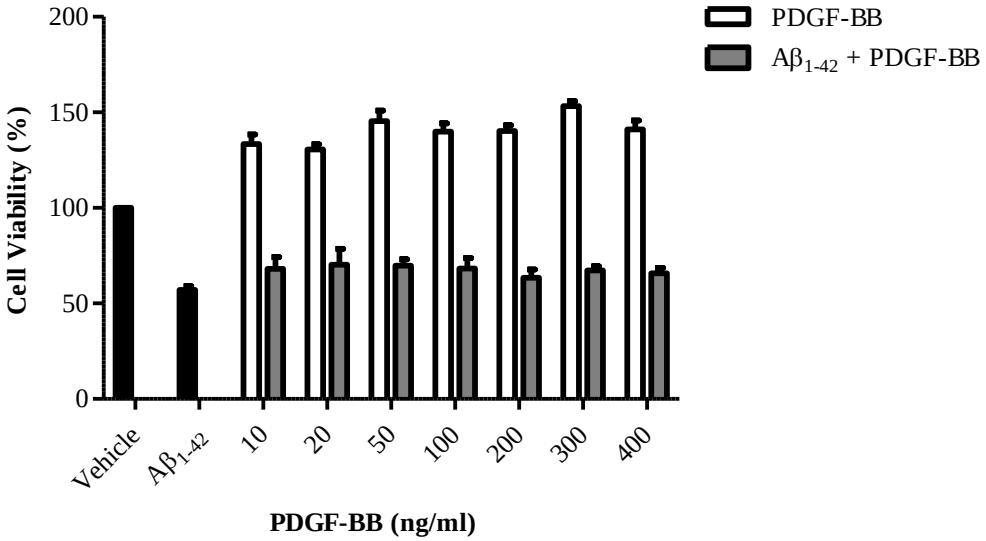


(A)

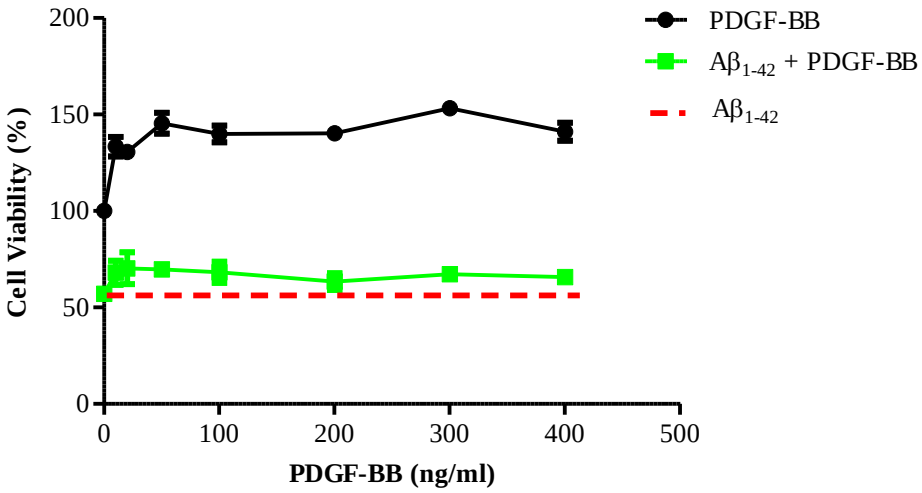


(B)

Figure 7: Neuroprotective effect of PDGF-BB at various concentrations against toxicity of $A\beta_{1-42}$ oligomer at 5 μM (co-treatment) in SH-SY5Y cells. (A) (B) SH-SY5Y cells (1×10^5/ml) were exposed to $A\beta_{1-42}$ oligomer at 5 μM in the presence or absence of PDGF-BB at various concentrations for 24hr. Cell viability was measured by MTT assay (n=4).

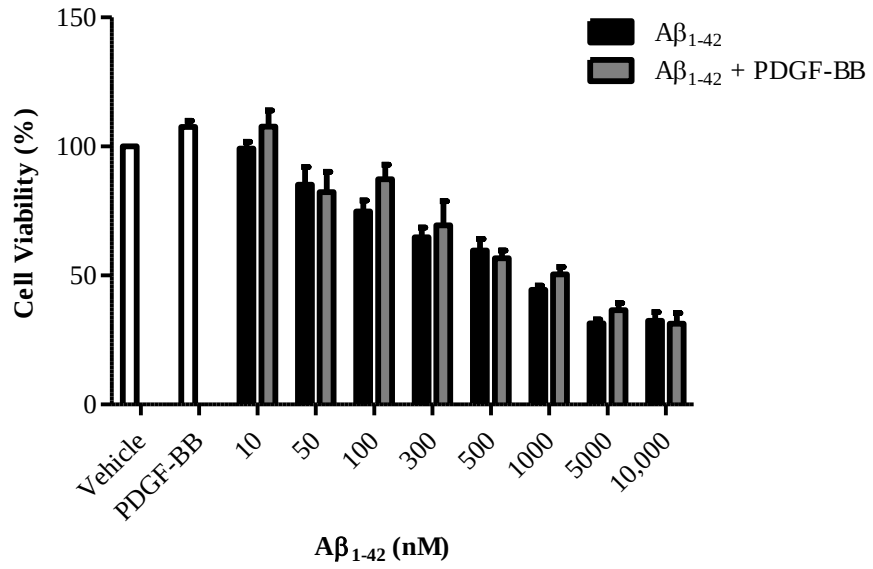


(A)

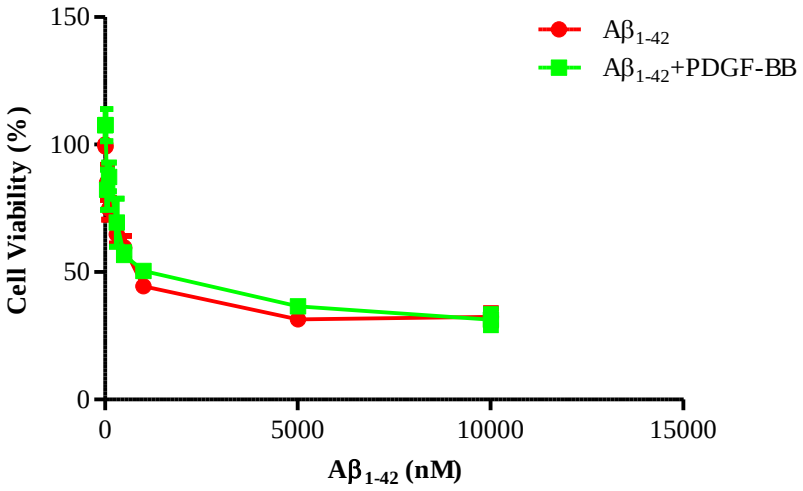


(B)

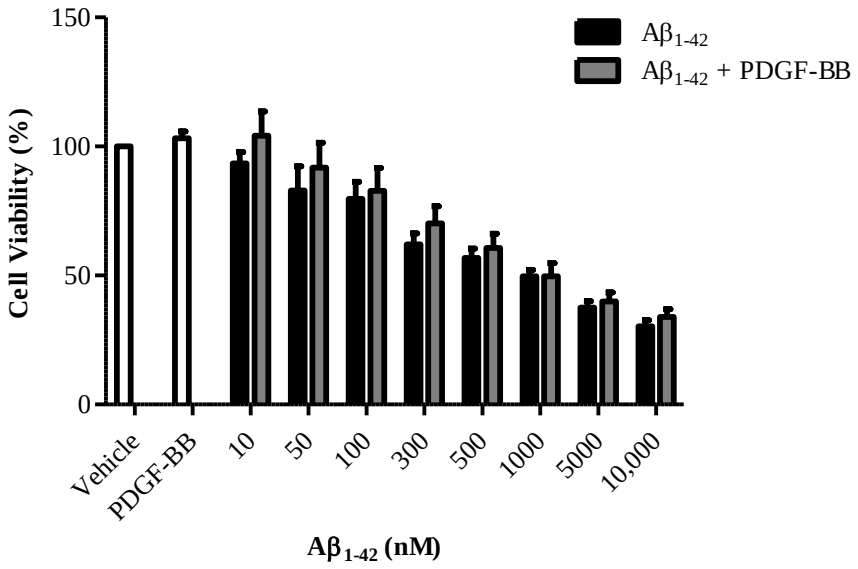
Figure 8: Neuroprotective effect of PDGF-BB at various concentrations against toxicity of $A\beta_{1-42}$ oligomer at 5 µM (PDGF-BB 30min pretreatment) in SH-SY5Y cells. (A) (B) SH-SY5Y cells (1×10^5/ml) were exposed to $A\beta_{1-42}$ oligomer at 5 µM for 24hr in the presence or absence of PDGF-BB at various concentrations that was added 30min before the exposure to $A\beta_{1-42}$. Cell viability was measured by MTT assay (n=5).


150
100
50
0
Cell Viability (%)
Aβ1-42
Aβ1-42 + PDGF-BB
Vehicle
PDGF-BB
10
50
100
300
500
1000
5000
10,000
Aβ1-42 (nM)

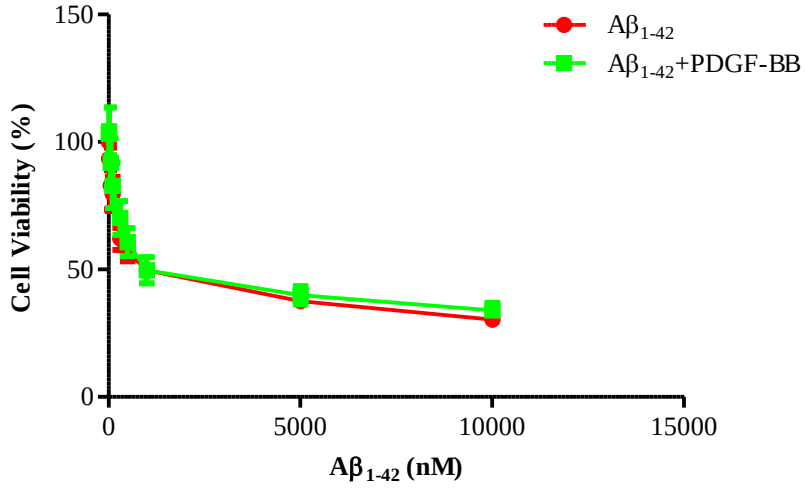
(A)


150
100
50
0
Cell Viability (%)
Aβ1-42
Aβ1-42+PDGF-BB
0
5000
10000
15000
Aβ1-42 (nM)

(B)

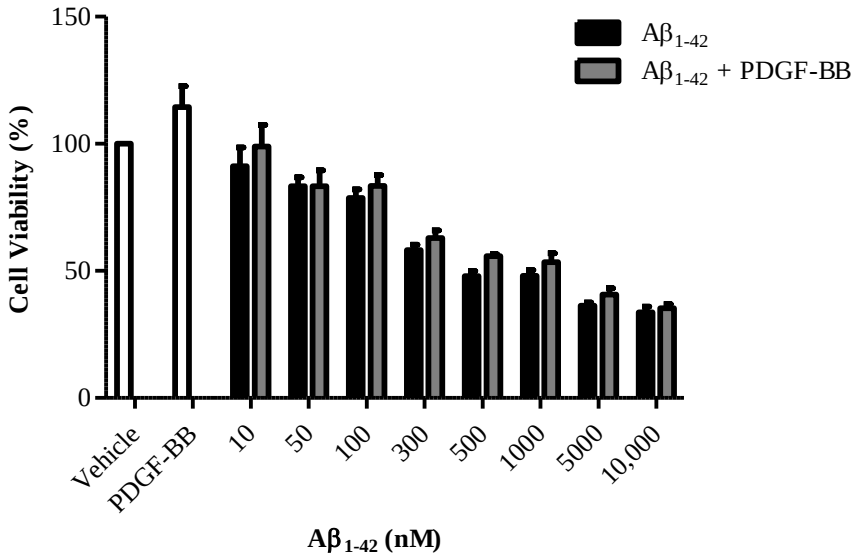


(C)

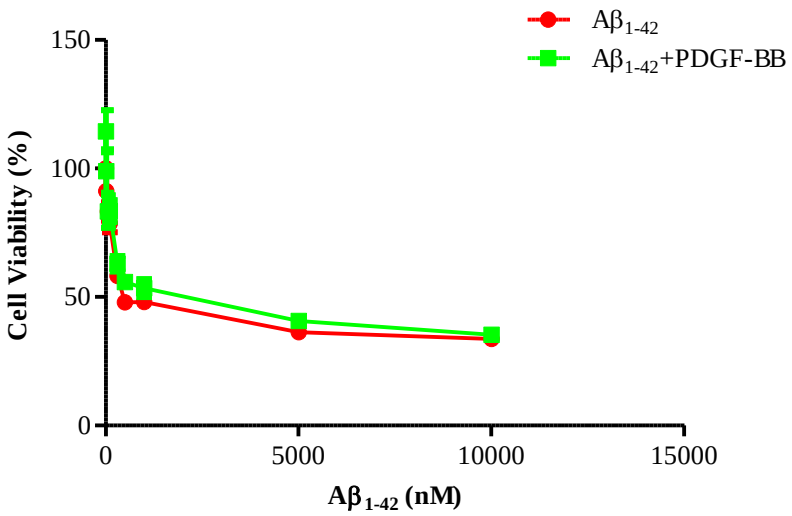


(D)

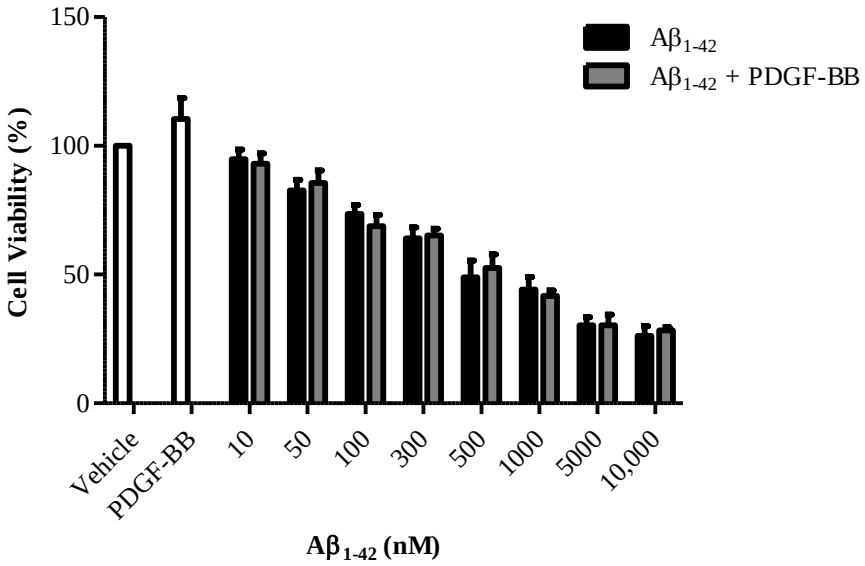
Figure 9: Neuroprotective effect of PDGF-BB at 10ng/ml against toxicity of Aβ1-42 oligomer at various concentrations (co-treatment) in primary neurons (DIV5). Embryonic mouse hippocampal neurons (DIV5) were exposed to Aβ1-42 oligomer at various concentrations in the presence or absence of PDGF-BB at 10 ng/ml for 24hr. Cell viability was measured by MTT assay. (A) (B) cell density = 0.5×10^5/ml (n=5); (C) (D) cell density = 1×10^5/ml (n=5).


150
100
50
0
Cell Viability (%)
Aβ1-42
Aβ1-42 + PDGF-BB
Vehicle
PDGF-BB
10
50
100
300
500
1000
5000
10,000
Aβ1-42 (nM)

(A)


150
100
50
0
Cell Viability (%)
Aβ1-42
Aβ1-42+PDGF-BB
0
5000
10000
15000
Aβ1-42 (nM)

(B)



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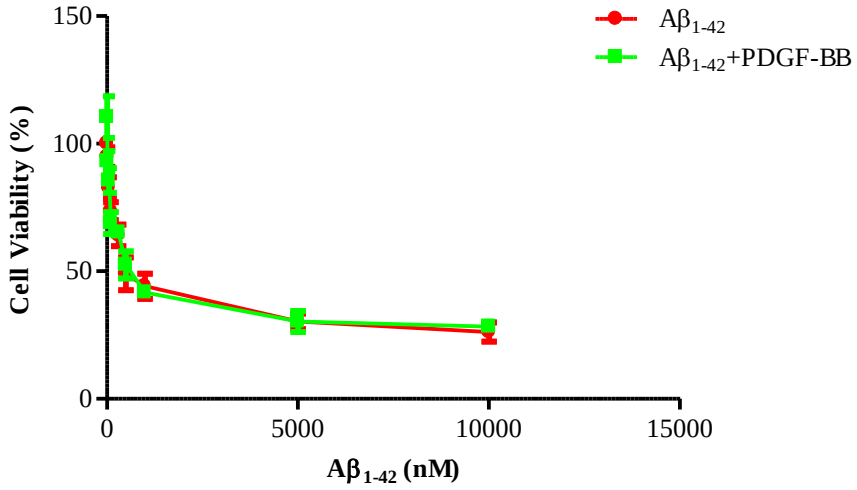
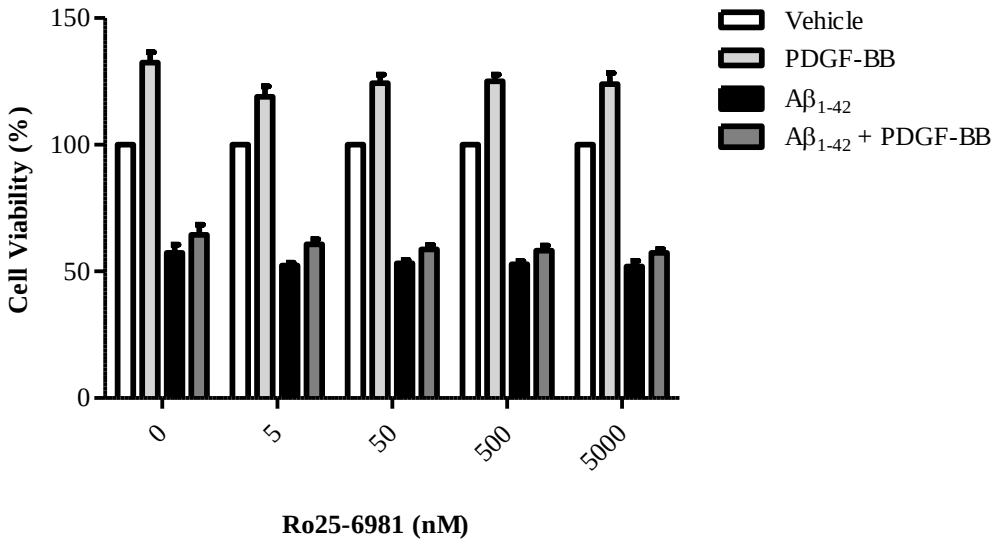
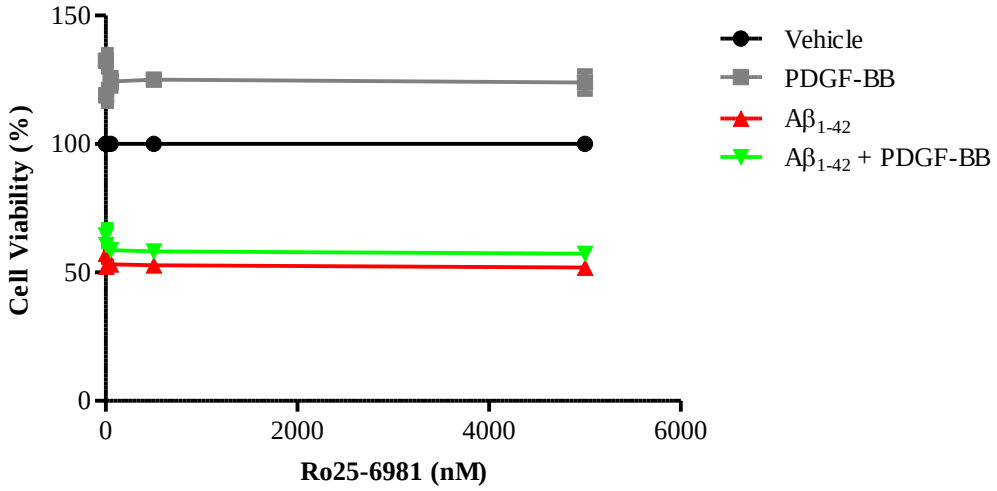


(D)

Figure 10: Neuroprotective effect of PDGF-BB at 10 ng/ml against toxicity of $A\beta_{1-42}$ oligomer at various concentrations (co-treatment) in primary neurons (DIV10). Embryonic mouse hippocampal neurons (DIV10) were exposed to $A\beta_{1-42}$ oligomer at various concentrations in the presence or absence of PDGF-BB at 10 ng/ml for 24hr. Cell viability was measured by MTT assay. (A) (B) cell density = 0.5×10^5/ml (n=5); (C) (D) cell density = 1×10^5/ml (n=4).



(A)



(B)

Figure 11: The involvement of NR2B containing NMDA receptor in PDGF-BB neuroprotective effect against $A\beta_{1-42}$ oligomer. (A) (B) SH-SY5Y cells (1×10^5/ml) were exposed to NR2B containing NMDA receptor antagonist Ro25-6981 at various concentrations immediately before the exposure to $A\beta_{1-42}$ oligomer at 5 μM and/or PDGF-BB at 10 ng/ml for 24hr. Cell viability was measured by MTT assay (n=7-8).

However, it might be too early to conclude that NMDA receptors are not involved in Aβ induced toxicity. A recent study (2011) shows $A\beta_{1-42}$ oligomer induced apoptotic cell death is dependent on ionotropic glutamate receptors including NMDA receptors: 24hr treatment of $A\beta_{1-42}$ oligomer in cultured cortical neurons induced dose-dependent cell death measured by LDH assay and this effect was attenuated by NMDA antagonists AP5, MK801 and memantine[83]. These results may be due to the many differences in the materials and methods being used, such as cell type, cell death assay and NMDA receptor antagonist.

SH-SY5Y cells have been used in numerous studies on NMDA receptors[84-87] and we have preliminary data from our laboratory showing the expression of NMDA receptors, including NR2B subunit, in this cell line. However, evidence for the functionality of the receptors in SH-SY5Y cells is contradictory. Some studies have shown the receptors can be activated by their agonists, such as NMDA and glutamate[88] and the NMDA receptor antagonist, MK801, is able to block the studied effect[87]; while others didn't see the receptor as functional[89, 90]. One explanation of the discrepancy could be the possibility of phenotype changes during passages in the tumor-originated cells. Therefore, it would be worthwhile to repeat the experiment in primary neurons in the future.

The MTT assay has been widely used in many similar neuroprotective studies against Aβ toxicity[78, 81, 91-93]. However, as indicated in the Aβ toxicity section in this report, MTT assay does have limitations reflecting many other effects that Aβ may cause, such as mitochondrial dysfunction[83, 94, 95], oxidative stress[76, 95], ATP depletion[76, 96], apoptosis[92, 93, 95], Ca^{2+} homeostasis disruption[83, 97] and neuronal morphological changes[97, 98]. The references on each of these Aβ toxic effects provide the corresponding quantitative and/or qualitative methods that should be considered among the limitations of using MTT assay alone in future studies.

The NR2B containing NMDA receptor has been implicated to be the major player of inducing excitotoxicity after neuronal insults[39]. The same NMDA receptor subtype is also implicated in having

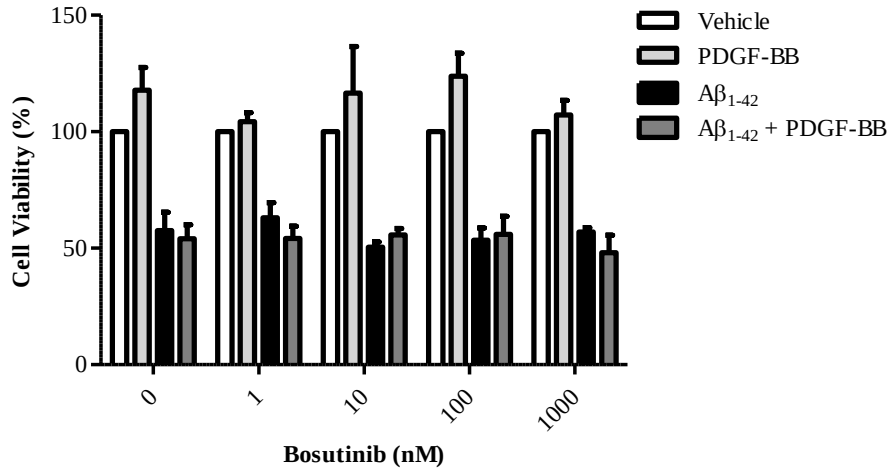
a pathological role in Alzheimer's disease[41, 80]. However, there is also a report showing the contradictory roles of these NMDA receptor subtypes: Texido and colleagues reported recently that $A\beta_{1-42}$ oligomer directly activated NMDA receptors with a preferential activation of NR2A-containing NMDA receptors compared to NR2B subtypes and that the antagonist of NR2B containing NMDA receptors, ifenprodil, only slightly attenuated $A\beta_{1-42}$ oligomer induced Ca^{2+} overload compared to the broad spectrum NMDA receptor antagonist, AP5[99]. Therefore, at the early stages of this study, it might be useful to start with NMDA receptor antagonists that are not specific for any subtypes, such as MK801, AP5 and memantine, prior to using subtype-selective antagonists.

Furthermore, it might be also too early to exclude PDGF neuroprotective effects against Aβ oligomers without testing Aβ oligomer induced synaptic dysfunction, another significant aspect of Aβ oligomer toxicity. Mounting evidence suggests that cognitive impairment in Alzheimer's disease starts with subtle alterations of synaptic function before neuronal loss becomes evident[100, 101]. Many studies have suggested soluble Aβ oligomers may affect synaptic function through NMDA receptors[40, 102]. A recent electrophysiology study (2011) shows that $A\beta_{1-42}$ oligomers can inhibit long-term potentiation (LTP) in murine hippocampal slices and NR2B containing NMDA receptor antagonists, including Ro25-6981, at 300nM, can reverse this effect[103]. This finding on the effects of NR2B containing NMDA receptor antagonists against Aβ oligomer induced synaptic deficits is in line with our previous findings that PDGF-BB is inhibitory on the same NMDA receptor subtype using the same method and antagonist[33]. Therefore, more thorough studies on Aβ oligomer induced synaptic dysfunction, especially the effect on LTP, will help clarify the protective role of PDGF-BB against Aβ oligomer toxicity from a new angle.

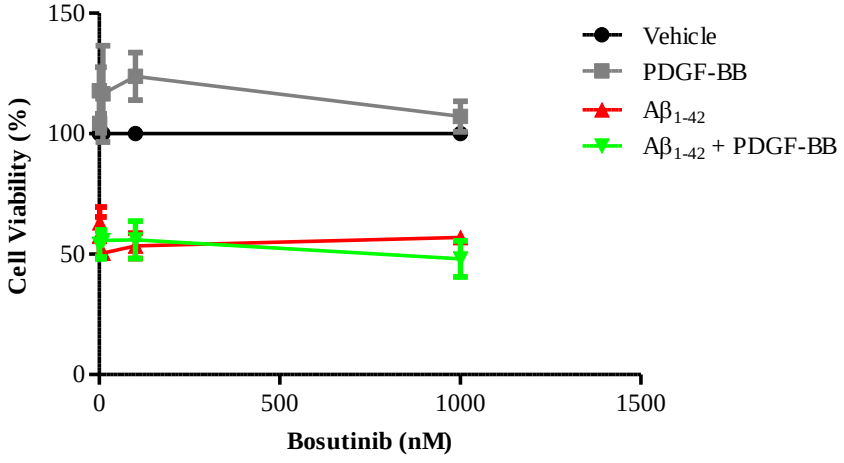
2.2.4. The involvement of c-Abl

Another axis of this study was to test the role of c-Abl. c-Abl is a downstream effector of PDGF β-receptor mediated signaling pathway[51] and plays a positive role in the PDGF inhibition on NMDA

receptors[32]. c-Abl is also implicated as a mediator in Aβ induced apoptosis[63, 64]. To determine the role of c-Abl in the proposed pathway, bosutinib, a c-Abl inhibitor was applied in the presence and absence of $A\beta_{1-42}$ oligomer and PDGF-BB. Figure 12 showed at all the tested dosages of bosutinib, neither the $A\beta_{1-42}$ oligomer induced toxicity nor the slight to no neuroprotective effect of PDGF-BB was altered.



(A)



(B)

Figure 12: The involvement of c-Abl in PDGF-BB neuroprotective effect against $A\beta_{1-42}$ oligomer. (A) (B) SH-SY5Y cells (1×10^5/ml) were exposed to c-Abl inhibitor bosutinib at various concentrations for 30min before the exposure to $A\beta_{1-42}$ oligomer at 5 μM and/or PDGF-BB at 10 ng/ml for 24hr. Cell viability was measured by MTT assay (n=8).

The unchanged Aβ oligomer induced toxicity by the addition of bosutinib is contradictory to the findings using another c-Abl inhibitor, STI571 (or imatinib) in the study of Aβ fibrils induced cell death[60, 64]. This might simply due to the two inhibitors used have slightly different targets. STI571, although has been widely used to inhibit c-Abl activity, has also been found to inhibit other tyrosine kinases such as PDGF receptors[104]; while bosutinib is a Src/Abl dual inhibitor[105] that lacks activity against PDGF receptors[104]. Another more plausible explanation is Aβ oligomer may elicit its toxicity through a different pathway that is not dependent on c-Abl as in Aβ fibril induced cell death.

Aβ fibrils and oligomers are quite different in shape and size. Since Aβ oligomer (or ADDL, amyloid-beta-derived diffusible ligand) induced toxicity became evident in recent studies, the preparations of the two species was defined in details and were often confirmed by AFM and/or Western blotting for their distinct sizes and shapes. Aβ fibrils are fiber-like protein aggregates (~4 nm in *z*-height and >1 μm in length)[106] with molecular weight in 50-100KDa[106]. They are mainly recognized as non-diffusible extracellular deposits[6]. In contrast, Aβ oligomers appear as globular structures (~2-6nm in *z*-height)[106,6] with the predominant species in the 17-42KDa[6] range comprising monomers, trimers, and tetramers[106]. These Aβ oligomers are soluble, membrane diffusible and have ligand-like features[6].

Although it is true that both species induce neurotoxicity, the magnitude and cellular response are quite different. Dahlgren and colleagues reported Aβ oligomer is 10-fold stronger than Aβ fibril in the inhibition of neuronal viability measured by MTT assay[106]. White and colleagues showed Aβ oligomer can induce inflammatory response more rapidly and potently than Aβ fibril[107]. Lee and colleagues found Aβ oligomer is more toxic than fibril in a negative effect on Akt activation[108]. Sondag and colleagues found $A\beta_{1-42}$ oligomer and fibril stimulate different signaling responses in microglia that lead to discrete effects on neuronal survival[109]. These findings imply that the two species may take different routes to neurotoxicity.

c-Abl is a non-receptor tyrosine kinase whose activity is strictly regulated: the kinase stays in an inactive form and becomes activated only in response to various stimuli. This strict regulation might be due to the fact that c-Abl can conduct distinct and even opposite signals, such as mediating both cell survival and cell death, depending on the nature of the stimuli. For example, c-Abl can response to growth factor stimuli and promote cell proliferation[50, 51]; while on the other hand, c-Abl can mediate apoptosis in response to DNA damage and oxidative stress[53]. In this investigation, the role of c-Abl in affecting cell viability is absent: there was no sign of suppressing or promoting cell viability (Figure 12). This could be due to the limitations of MTT assay that is only reflecting the change of metabolic activities in cells, which is not necessarily in close linkages to the activity of c-Abl in either cell growth or cell death pathways. Sims and colleagues found imatinib (STI571), another c-Abl inhibitor, can interfere with MTT assay and cause false results[110]. Therefore, other complementary cell viability assays should be used in future studies to corroborate the results.

2.2.5. A novel hypothesis

Besides the limitations of the experimental methods that may contribute to the lack of our expected results, an interesting observation throughout all the trials of the neuroprotection experiments may provide another explanation. As can be seen in Figure 5-12, in contrast to the poor protection elicited by PDGF-BB, $A\beta_{1-42}$ oligomer potently inhibited the neurotrophic effect induced by PDGF-BB. This observation not only provided a vital explanation on the slight to no neuroprotective effect induced by PDGF-BB, but also led to a novel hypothesis: Aβ oligomers may provoke neurotoxicity, at least partially, by inhibiting neurotrophic pathways. In order to test this hypothesis, a phosphorylation site, tyrosine 1021 (Tyr1021), on PDGF β-receptor was chosen to see if $A\beta_{1-42}$ oligomer can alter ligand-induced PDGF receptor phosphorylation. The results of this experiment were positive and may explain why PDGF-BB failed to protect neurons from $A\beta_{1-42}$ oligomer.

2.3. Aβ oligomer effects on PDGF receptor signaling

2.3.1. Phosphorylation of PDGF β-receptor at Tyr1021

PDGF-BB triggers neuroprotective pathways through binding to PDGF β-receptors[20, 27, 29]. The ligand binding results in receptor dimerization and activation of the receptor's intrinsic kinase activity. As a result, receptor trans-phosphorylation occurs at multiple tyrosine residues which in turn become binding sites to recruit molecules that will transduce signals further. The residue Tyr1021 near the C-terminal of the receptor is one of the sites activated during trans-phosphorylation upon ligand binding and was chosen as the first site to investigate the influence of Aβ oligomer on PDGF β receptor activation.

The investigation began by repeating the same conditions as in the MTT assay: SH-SY5Y cells were treated with $A\beta_{1-42}$ oligomer at 5 μM and PDGF-BB at 10 ng/ml together for 24hr. The result shows that $A\beta_{1-42}$ oligomer exhibited a potent inhibition on PDGF-BB induced PDGF β-receptor phosphorylation at Tyr1021: the co-treatment decreased the phosphorylation by about 1/3 (Figure 13).

In order to clarify this inhibition by $A\beta_{1-42}$ oligomer, the initial effect of PDGF-BB on the receptor phosphorylation was excluded by incubating the cells with $A\beta_{1-42}$ oligomer alone for 24hr first followed by stimulating the phosphorylation by PDGF-BB for just 10min before the cells were lysed. As expected, the inhibitory effect became more apparent: 24hr treatment by $A\beta_{1-42}$ oligomer at 5 μM reduced PDGF-BB induced PDGF β-receptor phosphorylation at Tyr1021 by more than 2/3 (Figure 14).

Then the investigation was pushed even further by asking if a simple, acute treatment of $A\beta_{1-42}$ oligomer can cause the same inhibitory effect. Cells were treated with $A\beta_{1-42}$ oligomer for only 10min before being stimulated by PDGF-BB. Interestingly, this acute treatment of $A\beta_{1-42}$ treatment at the

same concentration (5 μM) was enough to block the PDGF-BB induced PDGF β-receptor phosphorylation at Tyr1021 by more than half (Figure 15).

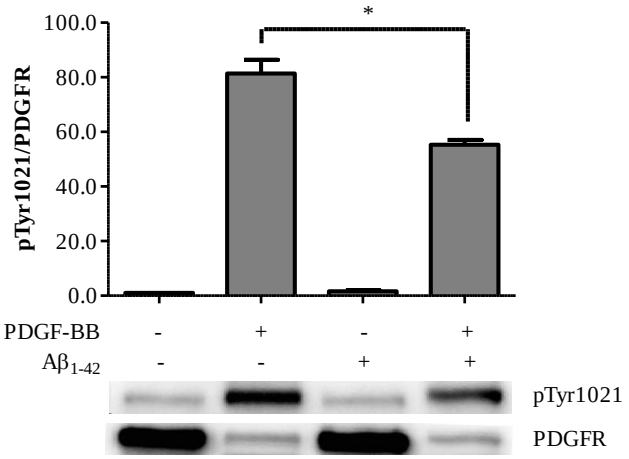


Figure 13: $A\beta_{1-42}$ oligomer treatment for 24hr suppressed PDGF-BB induced PDGF β-receptor phosphorylation at Tyr1021 ($A\beta_{1-42}$ and PDGF-BB co-treatment). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 24hr in the presence or absence of PDGF-BB at 10 ng/ml. Western blot membranes were probed with phospho-PDGF β-receptor (Tyr1021) antibody, stripped, and re-probed with PDGF β-receptor antibody. Blots are representative of 4 independent experiments. * $p < 0.05$, t-test.

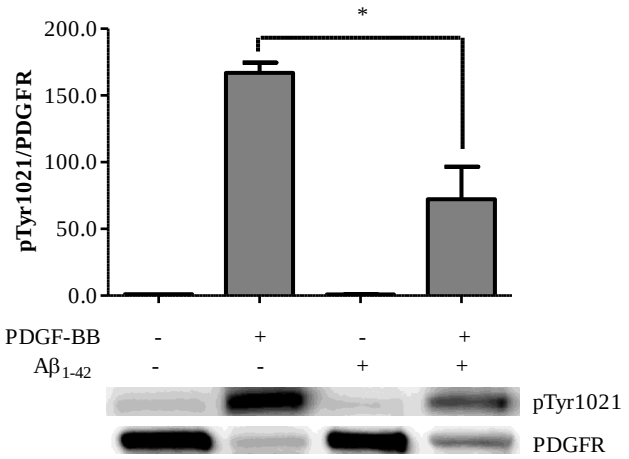


Figure 14: $A\beta_{1-42}$ oligomer treatment for 24hr suppressed PDGF-BB induced PDGF β-receptor phosphorylation at Tyr1021 (PDGF-BB 10min stimulation). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 24hr before stimulated by PDGF-BB at 10 ng/ml for 10min. Western blot membranes were probed with phospho-PDGF β-receptor (Tyr1021) antibody, stripped, and re-probed with PDGF β-receptor antibody. Blots are representative of 3 independent experiments. * $p < 0.05$, t-test.

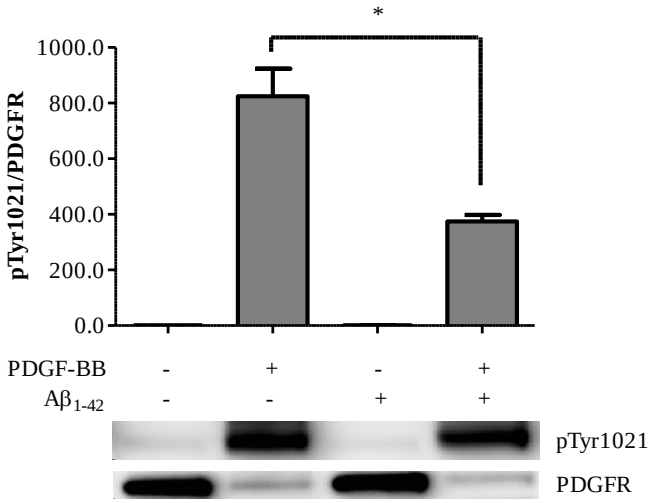


Figure 15: $A\beta_{1-42}$ oligomer acute treatment for 10min suppressed PDGF-BB induced PDGF β-receptor phosphorylation at Tyr1021 (PDGF-BB 10min stimulation). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 10min before stimulated by PDGF-BB at 10 ng/ml for 10min. Western blot membranes were probed with phospho-PDGF β-receptor (Tyr1021) antibody, stripped, and re-probed with PDGF β-receptor antibody. Blots are representative of 3 independent experiments. * $p < 0.05$, t-test.

These findings provided an explanation for the results obtained from MTT assays. Phosphorylated Tyr1021 is the major binding site of PLC-γ[111], a 145-kDa protein that catalyzes the hydrolysis of a phospholipid, phosphatidylinositol 4,5-bisphophate (PIP_2) and leads to the production of two secondary messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG)[112]. IP3 is known to stimulate internal Ca^{2+} release from endoplasmic reticulum (ER) and DAG can work with the increased Ca^{2+} to activate protein kinase C (PKC), a serine and threonine kinase[112] that plays a role in triggering cell proliferation and differentiation[113]. Phosphorylation at Tyr1021 is required for a stable association of the receptor with PLCγ, PDGF-dependent IP_3 production, and maximal DNA synthesis, a mitogenic response induced by PDGF-BB[25]. Therefore, the poor neuroprotective effect of PDGF-BB against $A\beta_{1-42}$ oligomer and the potent inhibitory effect of $A\beta_{1-42}$ oligomer on PDGF-BB induced promotion on cell survival that consistently appeared throughout all the MTT assays may be due to the fact that PDGF-BB's effects were inhibited by $A\beta_{1-42}$ oligomers throughout these experiments.

These findings also partially supported our novel hypothesis that Aβ oligomers may elicit their neurotoxicity through inhibition on neurotrophic pathways (Figure 16). Neurotrophic factors (NTFs) play important roles in both developing and mature nervous systems. Although the production is declined after the early development, neurotrophic factors continue to nurture adult brains by maintaining healthy neuronal populations for optimal survival and function[114, 115]. Many studies have shown that NTF function may be disturbed in neurodegenerative diseases including Alzheimer's disease: the levels of some NTFs and their associated receptors were found being altered in AD brains and there are strong association between some NTFs and the formation of neurofibrillary tangles and senile plaques[116-118]. However, less attention has been paid to the mechanism of how NTF function is affected. The findings in this study on the inhibitory effect of Aβ oligomer on PDGF receptor activation may provide some insights into the alteration of NTF function in AD. The altered levels of NTFs and NTF receptors might be due to the strong inhibition of Aβ oligomers on NTF receptor activation and the affected brains may attempt to express more NTFs and/or NTF receptors to foster the activation. Since NTF signaling pathways may be targeted by Aβ oligomers, it is not surprising to see the presence of NTFs at the sites where Aβ oligomers start to accumulate to form senile plaques or start to stimulate tau hyperphosphorylation and neurofibrillary tangle formation.

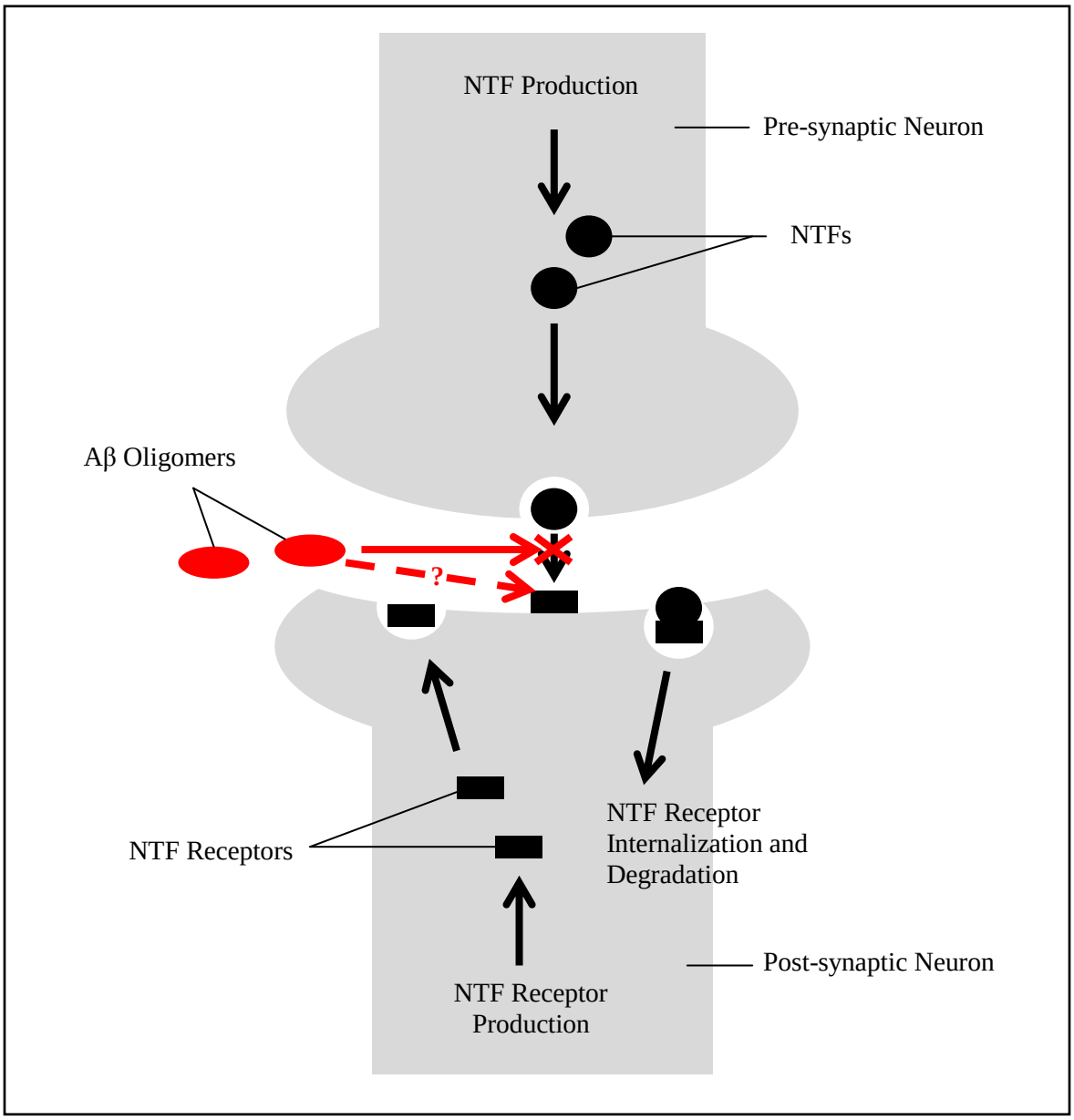


Figure 16: A novel hypothesis of Aβ oligomer induced toxicity in Alzheimer's disease. Aβ oligomers may elicit their toxicity through inhibition on neurotrophic factor (NTF) signaling pathways that are essential for maintaining healthy neuronal population in the mature central nervous system. The activation of NTF receptors by their ligands is blocked by Aβ oligomers possibly through a way that Aβ oligomers may act as antagonists of the receptors.

2.3.2. Other effects of $A\beta_{1-42}$ oligomer on PDGF β-receptor signaling

Some efforts were made in this project to elucidate the Aβ oligomer inhibition mechanism by examining the level of PDGF β-receptor, the phosphorylation and expression of extracellular-signal regulated kinase 1/2 (Erk1/2) in SH-SY5Y cells.

Treatment of $A\beta_{1-42}$ oligomer for 24hr didn't change the level of PDGF β-receptor comparing to the control (Figure 17 &18). This finding excluded the possibility that $A\beta_{1-42}$ oligomer may affect PDGF receptor signaling by regulating the receptor expression. Interestingly, co-treatment of $A\beta_{1-42}$ oligomer with PDGF-BB for 24hr didn't block the down-regulation of the receptor induced by its ligand (Figure 17); while when the cells were pre-exposed to $A\beta_{1-42}$ oligomer for 24hr before the PDGF-BB stimulation for 10min, there was a slight but significant reduction of the down-regulation (Figure 18); this effect was not seen in the acute exposure of $A\beta_{1-42}$ oligomers (Figure 19).

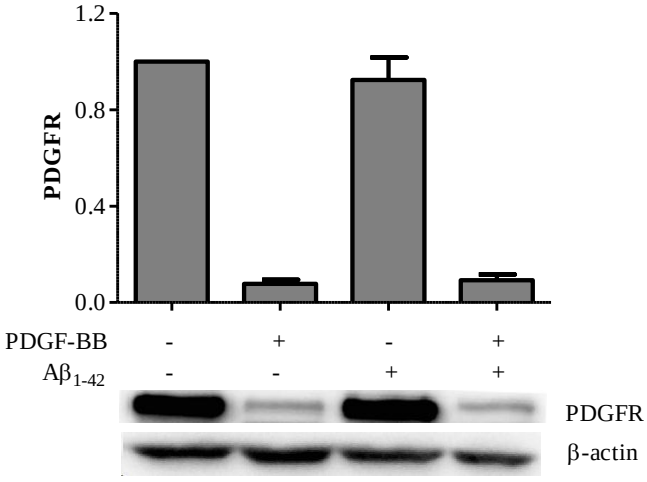


Figure 17: $A\beta_{1-42}$ oligomer treatment for 24hr didn't alter the protein level of PDGF β-receptor ($A\beta_{1-42}$ and PDGF-BB co-treatment). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 24hr in the presence or absence of PDGF-BB at 10 ng/ml. Western blot membranes were probed with PDGF β-receptor antibody, stripped, and re-probed with β-actin antibody as a loading control. Blots are representative of 7 independent experiments.

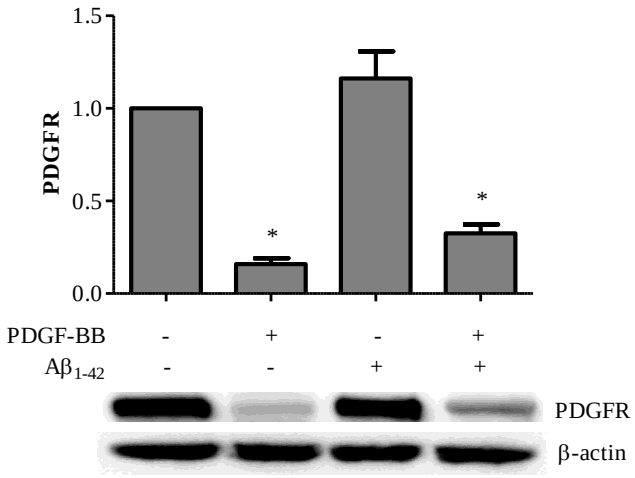


Figure 18: $A\beta_{1-42}$ oligomer treatment for 24hr slightly hindered PDGF-BB induced PDGF β-receptor down-regulation (PDGF-BB 10min stimulation). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 24hr before stimulated by PDGF-BB at 10 ng/ml for 10min. Western blot membranes were probed with PDGF β-receptor antibody, stripped, and re-probed with β-actin antibody as a loading control. Blots are representative of 3 independent experiments. * $p < 0.05$, t-test.

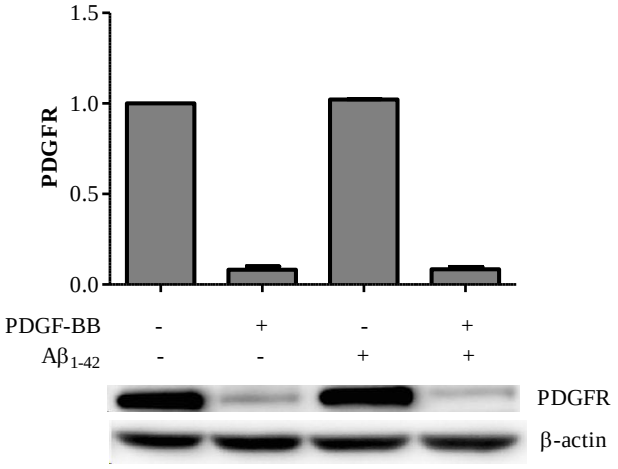


Figure 19: $A\beta_{1-42}$ oligomer acute treatment didn't affect PDGF-BB induced PDGF β-receptor degradation. SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 10min before stimulated by PDGF-BB at 10 ng/ml for 10min. Western blot membranes were probed with PDGF β-receptor antibody, stripped, and re-probed with β-actin antibody as a loading control. Blots are representative of 4 independent experiments.

The ligand induced receptor down-regulation is mediated through receptor internalization and degradation, which is common among many receptor tyrosine kinases[119-121]. It was found that activation of PDGF receptors promotes the degradation but is not required[122]. It is possible that $A\beta_{1-42}$ oligomer only partially affected the activation of the receptors and the receptor internalization and degradation was induced by its ligand very quickly before being affected by $A\beta_{1-42}$ oligomer. Besides, the slight change of the receptor degradation required long-time exposure of $A\beta_{1-42}$ oligomer.

Erk is an important downstream effector of PDGF-induced mitogenesis. Evidence has shown Erk may mediate the neuroprotective effect elicited by PDGF-BB against HIV1 Tat induced cytotoxicity in the context of HIV-associated dementia (HAD)[21] and against H_2O_2 induced cytotoxicity[20] that mimics the oxidative stress happened in many neurological disorders, including Alzheimer's disease[123]. Therefore, it was of interest to examine if Erk could be affected by Aβ oligomer treatment.

As can be seen in Figure 20-22, $A\beta_{1-42}$ oligomer didn't change the basal or the induced levels of Erk phosphorylation in all three conditions: $A\beta_{1-42}$ oligomer and PDGF-BB co-treatment for 24hr (Figure 20), $A\beta_{1-42}$ oligomer pre-treatment for 24hr before PDGF-BB stimulation for 10min (Figure 21), and $A\beta_{1-42}$ oligomer acute treatment for 10min before PDGF-BB stimulation for 10min (Figure 22). Meanwhile, the protein level of Erk was not altered by the treatment of $A\beta_{1-42}$ oligomer for 24hr (Figure 23). These findings indicate that $A\beta_{1-42}$ oligomer may not affect Erk activation at the basal level or simulated level induced by PDGF-BB and has no impact on Erk protein level within 24hr.

These findings stand in contrast to other studies that suggest that $A\beta_{1-42}$ oligomers may affect the basal level of Erk activation in a time-dependent manner: in one study conducted by Young and colleagues, $A\beta_{1-42}$ oligomer at 100 nM alone increased Erk1/2 phosphorylation that peaked at 10min but declined to basal level after 30-120min in SH-SY5Y cells[124]; in another study performed by Ma and colleagues, $A\beta_{1-42}$ oligomer decreased phosphorylation of Erk2 at 1.5hr and 24hr in SH-SY5Y cells and this effect can be reversed by Aβ oligomer-specific antibody[125]. In day 7 hippocampal slice

culture, Bell and colleagues observed a slight but significant increase of Erk2 phosphorylation induced by oligomeric $A\beta_{1-42}$ at 100 nM for 5min but the Erk2 phosphorylation became fluctuant around the basal level when the culture was treated with the same amount of $A\beta_{1-42}$ oligomer for extended time (2-16hr)[126].

Since the level of active Erk is not steady but rather fluctuant in response to $A\beta_{1-42}$ oligomers, it is possible that the change of Erk phosphorylation from basal level induced by $A\beta_{1-42}$ oligomer was simply missed in my experiment. For example, in the case of the Aβ acute treatment (Figure 15), there was an additional 10min for PDGF-BB after the cells were incubated with $A\beta_{1-42}$ for 10min and therefore when the cells were lysed, they had been exposed to $A\beta_{1-42}$ for 20min and the level of phosphorylated Erk1/2 could have returned to the basal level already as indicated in Young's study. The different concentration of $A\beta_{1-42}$ oligomer being used (5 μM vs. 100 nM) may also contribute to the discrepancies. Since it has been postulated that Erk activation may play a role in Aβ oligomer induced neurotoxicity[124-127], it might be worthwhile to conduct a time-course experiment from 5min in both SH-SY5Y cells and primary neurons to reveal a clearer picture on how $A\beta_{1-42}$ oligomers may affect the basal level of Erk phosphorylation.

To continue the research conducted in this project that aimed to discover the effect of Aβ oligomers on the signaling pathways induced by PDGF and other neurotrophic factors in the future, there are many other studies should be done than testing Erk phosphorylation alone. For example, a screening on other phosphorylation sites on PDGF receptors can be done quickly. This simple and fast experiment will provide a clearer picture on how PDGF receptor activation being affected by Aβ oligomers and what specific phosphorylation sites are affected. From there, the corresponding pathways can be examined more specifically. One pathway that might worth special attention is phosphatidylinositol-3-kinase (PI3K)/Akt pathway since it has been strongly implicated in Aβ oligomer induced toxicity[108, 128], glycogen synthase kinase-3 (GSK-3) associated tau pathology[129, 130]

as well as PDGF neuroprotection[20, 22, 131]. Furthermore, other neurotrophic factors that have been strongly implicated in Alzheimer's disease should be tested. For example, nerve growth factor (NGF) family NTFs, including NTF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NF-3) and neurotrophin-4/5 (NF-4/5) have been studied most intensively in AD related research[114, 117, 132] and NGF has been used in clinic trial to treat Alzheimer's disease[133]. Thus, a similar study as in this project using NGF or BDNF may be able to reveal if $A\beta_{1-42}$ oligomer also has a strong negative impact on neuronal regeneration process induced by these factors; if it is true, the result will shed light on improving the efficiency of using these NTFs as therapeutic agents to treat Alzheimer's disease.

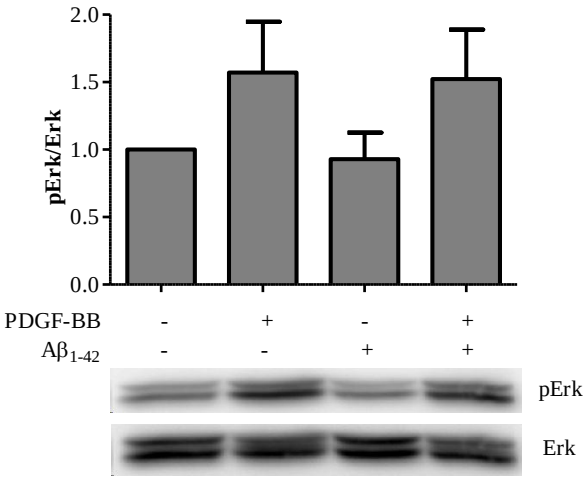


Figure 20: $A\beta_{1-42}$ oligomer treatment for 24hr didn't affect Erk phosphorylation at both basal and stimulated level ($A\beta_{1-42}$ and PDGF-BB co-treatment). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 24hr in the presence or absence of PDGF-BB at 10 ng/ml. Western blot membranes were probed with phospho-Erk antibody, stripped, and re-probed with Erk antibody. Blots are representative of 3 independent experiments.

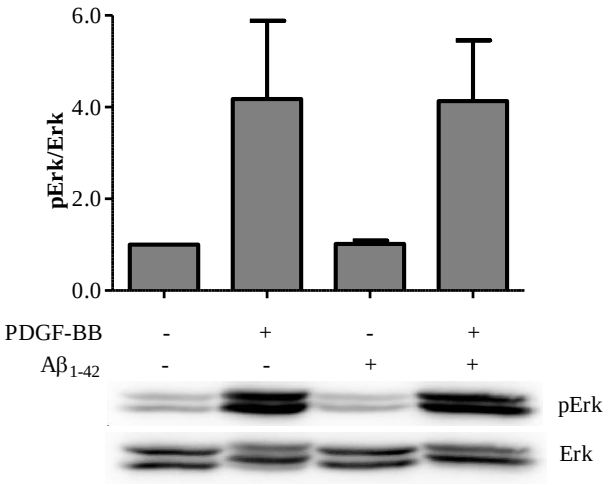


Figure 21: $A\beta_{1-42}$ oligomer treatment for 24hr didn't affect Erk phosphorylation at both basal and stimulated level (PDGF-BB 10min stimulation). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 24hr before stimulated by PDGF-BB at 10 ng/ml for 10min. Western blot membranes were probed with phospho-Erk antibody, stripped, and re-probed with Erk antibody. Blots are representative of 4 independent experiments.

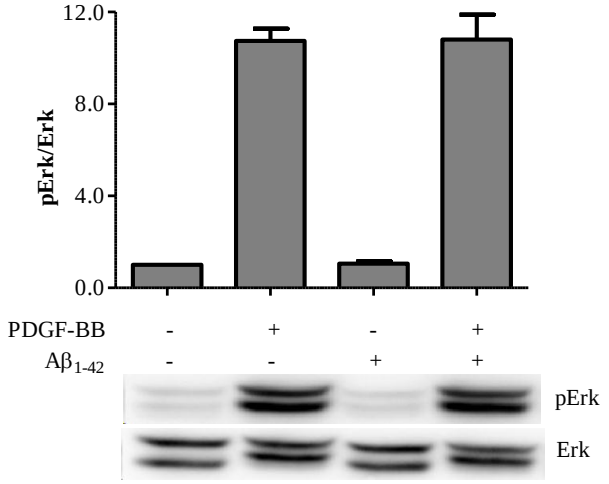


Figure 22: $A\beta_{1-42}$ oligomer acute treatment for 10min didn't affect Erk phosphorylation at both basal and stimulated level (PDGF-BB 10min stimulation). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 10min before stimulated by PDGF-BB at 10 ng/ml for 10min. Western blot membranes were probed with phospho-Erk antibody, stripped, and re-probed with Erk antibody. Blots are representative of 4 independent experiments.

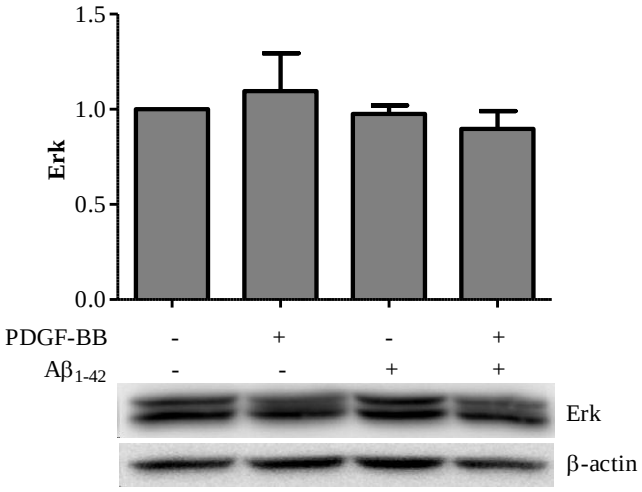


Figure 23: $A\beta_{1-42}$ oligomer treatment for 24hr didn't affect Erk expression ($A\beta_{1-42}$ and PDGF-BB co-treatment). SH-SY5Y cells were exposed to $A\beta_{1-42}$ oligomer at 5 μM for 24hr in the presence or absence of PDGF-BB at 10 ng/ml. Western blot membranes were probed with Erk antibody, stripped, and re-probed with β-actin antibody as a loading control. Blots are representative of 3 independent experiments.

Chapter III

Materials and Methods

3.1. $A\beta_{1-42}$ oligomer preparation

This is a modified version of Stine's protocol[67]. Briefly, $A\beta_{1-42}$ (rPeptide, Georgia, USA) was dissolved in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Sigma) to 1 mg/ml solution and aliquoted. HFIP was allowed to evaporate in a chemical fume hood until no visible liquid was left in the tube. The resulting peptide film was dried under vacuum for 1hr and stored in a glass desiccator at -20°C. Immediately prior to use, the film was re-suspended to 5 mM in DMSO with 10min sonication at room temperature, diluted into 100 µM in DMEM/F12 serum and phenol red free medium and incubated at 4°C for 24hr.

3.2. SH-SY5Y cell culturing

The SH-SY5Y human neuroblastoma cell line is a generous gift from Dr. Shilpa Buch and colleagues (University of Nebraska Medical Center, USA). The cells were maintained in DMEM/F-12 (1:1) with L-glutamine and HEPES (Fisher) supplemented with 10% fetal bovine serum (FBS) (Sigma) in a humid atmosphere of 5% CO_2 at 37°C. Prior to treatment with reagents, cells were serum starved for 24hr by using the same medium without supplementation of FBS. For MTT assay, the same medium without phenol red (Fisher) was used for the serum starvation.

3.3. Primary neuronal cell culturing

Primary neuronal cell cultures were produced from hippocampi of 17-day mouse embryos (E17). Animal care and treatment were in accordance with institutional guidelines. The time-pregnant mouse (E17) was sacrificed by cervical dislocation. The fetuses were then removed and placed in cold

dissection medium (15 mL HEPES, 500 mL HBSS, 2.5 g glucose (0.6%), 10 g sucrose (2%), pH 7.4, 320-335 mOsm/kg). The hippocampi were separated from the brains under a microscope and digested in 0.25% trypsin/0.1% EDTA for 30min at 37°C. Following the removal of the trypsin/EDTA, the cells were rinsed with 1 ml plating medium (DMEM-high glucose, supplemented with 10% FBS, 10% horse serum) (Sigma) for two times and homogenized into 1 ml plating medium by trituration using a pipette. The resulting cell mixture was filtered through a cell strainer (BD) and diluted into 1/10 hippocampus per ml of plating medium. Cells were counted and plated on poly-D-lysine (Sigma) coated 96-well plates in plating medium and grown at 37°C in a humidified atmosphere containing 5% CO_2. After 2-4hr or until cell attachment is established, 50 µl of the plating medium was exchanged to feeding medium (serum-free B-27 supplemented Neurobasal medium (Invitrogen) with 1% penicillin/streptomycin (Fisher)) in each well. Three days after plating, FUDR (0.081 mM 5-fluoro-2-deoxyuridine and 0.2 mM uridine in DMEM-high glucose) (sigma), a mitotic inhibitor, was added to inhibit the growth of non-neuronal cells. After 24hr of FUDR treatment, 30 µl of the medium was exchanged for 30 µl of fresh feeding medium and this same medium change strategy was applied for all the medium change thereafter. Treatment with reagents was performed 5 or 10 days after plating. Medium was changed on day 6 and day 9 for the plates treated on day 10.

3.4. MTT assay

Cell viability was measured by quantitative colorimetric assay with MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide), showing the MTT reduction by viable cells as described in published literatures[65, 134]. MTT (Sigma) stock solution (5 mg/ml) was prepared by dissolving MTT into phosphate buffered saline (PBS). The solution was filtered, aliquoted and stored at -20°C in amber microcentrifuge tubes. Each experimental condition was added to 3 wells of the 96-well plates. After treatment, the MTT stock solution at a volume of 10% of the medium volume was added per well and incubated for 3hr. The resulting formazan crystals were dissolved by adding an amount of

MTT solubilization solution (10% Triton X-100 and 0.1 N HCl in anhydrous isopropanol) (Sigma) equal to the total volume of original medium followed by trituration. Absorbance was measured at 570nm and subtracted by background absorbance at 690nm. For each wavelength, a blank was used. Cell viability was expressed as percentage of absorbance relative to the cells treated with vehicle.

3.5. Western blotting

After treatment with reagents, cells were washed with chilled PBS, and lysed in chilled lysis buffer (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1% triton-X; supplemented with Halt Protease and Phosphatase Inhibitor (Fisher) prior to use). Cells were scraped, homogenized and centrifuged at 13,000 x g for 20min at 4°C and the supernatant was collected. Homogenates were subjected to SDS-PAGE and proteins were transferred to nitrocellulose membranes, blocked with 5% non-fat dry milk in Tris-buffered saline and 0.1% Tween-20 (TBST) for 1hr at room temperature or overnight at 4°C, and incubated in primary antibodies for 1hr at room temperature or overnight at 4°C. Membranes were washed three times in TBST, incubated with HRP-conjugated secondary antibodies for 1hr at room temperature, washed again, and bound antibodies were visualized by enhanced chemiluminescence using chemiluminescent substrate Luminata Crescendo HRP (Fisher). Images of Western blots were taken on a Kodak 4000MM Pro Imaging Station, and densitometric analyses were performed using Kodak Molecular Imaging software. Membranes were then stripped and re-probed with other antibodies. Primary antibodies used include those raised against PDGF β-receptor (Santa Cruz), phospho-PDGF β-receptor at Tyr 1021 (Santa Cruz), Erk (New England Biolabs), Phospho-Erk (New England Biolabs), and β-actin (Santa Cruz).

Chapter IV

Conclusions

The optimization steps taken at the beginning in this project provided a solid methodological foundation for generating reproducible data in this project as well as to establish this technique in the lab for future experimentation. The optimized $A\beta_{1-42}$ oligomer preparation and the established four-day MTT assay routine in this project were proven to result in consistent results in both SH-SY5Y cells and primary neurons. These optimized procedures can be applied in many other studies testing neuroprotective agents against Aβ oligomer toxicities in SH-SY5Y cells and primary neurons.

$A\beta_{1-42}$ oligomer elicited a dose-dependent toxicity with a sign of saturation at the higher dosages in both SH-SY5Y cells and primary neurons as measured by MTT assay. The saturation suggested a possible Aβ-receptor binding mechanism behind $A\beta_{1-42}$ oligomer induced toxicity. Additional assays that test other aspects of Aβ toxicity should be used to describe Aβ oligomer toxicity in a more comprehensive way in the future.

PDGF-BB showed a slight to no neuroprotective effects against $A\beta_{1-42}$ oligomer induced toxicity in MTT assay for both SH-SY5Y cells and primary neurons and NR2B-containing NMDA receptor was not involved. The limitation of the MTT assay, the cancerous nature of SH-SY5Y cell line and the usage of the specific NMDA receptor antagonist may contribute to the unexpected results. Using additional assays to corroborate the MTT assay results, applying broader spectrum NMDA receptor antagonists, testing the involvement of NMDA receptors in primary neurons and examining the synaptic dysfunction caused by Aβ oligomer in the future may provide a clearer picture on the perceived PDGF neuroprotective effects and the involvement of NMDA receptors in Aβ oligomer induced neurotoxicity.

MTT assay in SH-SY5Y cells using c-Abl inhibitor showed that c-Abl was neither promoting nor inhibiting PDGF-BB's neuroprotective effects against $A\beta_{1-42}$ oligomer induced cytotoxicity. The discrepancy compared to previous reported role of c-Abl may be explained by the usage of different inhibitors, the distinct features of Aβ oligomers, and the limitations of the MTT assay.

The inhibitory effects of $A\beta_{1-42}$ oligomer on PDGF-BB induced mitogenesis observed in MTT assay for both SH-SY5Y cells and primary neurons not only explained the poor neuroprotective effect elicited by PDGF-BB against $A\beta_{1-42}$ oligomer but also led to a novel hypothesis that Aβ oligomers may elicit neurotoxicity through inhibition on neurotrophic factor induced neuronal regeneration pathways.

This novel hypothesis was supported by results from Western blotting analysis: PDGF-BB induced PDGF β-receptor phosphorylation at Tyr1021 can be potently suppressed by $A\beta_{1-42}$ oligomer both acutely and in 24hr. Further Western blotting analyses on PDGF β-receptor levels, Erk phosphorylation and expression suggested that $A\beta_{1-42}$ oligomer may only partially inhibit PDGF β-receptor activation and its downstream pathways. Future investigation on other phosphorylation sites of PDGF receptor and other signaling pathways downstream of PDGF receptor activation will provide a clearer picture on how Aβ oligomers affect PDGF receptor signaling. Besides, the same strategies developed in this study can also be used to examine the effect of Aβ oligomers on other neurotrophic factor signaling systems.

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