Identifying the mechanism of Aβ42 inhibition of PDGF-BB signalling

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:

Alzheimer's disease is a late-onset neurological disorder characterized by extracellular aggregates of Aβ plaques and neurofibrillary tangles of hyperphosphoryated tau protein resulting in neuronal dysfunction, cognitive decline and death. Some of the molecular mechanisms which cause neuronal dysfunction in Alzheimer's disease are oxidative stress, excitotoxicity and hypoxia. PDGF-BB is a neurotrophic factor which is neuroprotective against these molecular events. However, PDGF-BB failed to be neuroprotective against Aβ42 toxicity in SH-SY5Y neuroblastoma cells. Rather, Aβ42 actually inhibited PDGF-BB signalling and reduced the phosphorylation level at multiple phosphotyrosine sites on the PDGFβ receptor. Aβ42 inhibition of PDGF-BB signalling also inhibited a downstream effector, Akt, a neuroprotective protein. Thus, Aβ42 inhibition of PDGF-BB signalling could worsen oxidative stress, excitotoxicity and hypoxia observed in Alzheimer's disease. Indeed, Aβ42 treatment prevented PDGF-BB neuroprotection against excitotoxicity. Aβ42 mediated inhibition of PDGF-BB signalling was not due to Aβ42 interaction with PDGFβ receptor. However, it remains inconclusive whether Aβ42 binds to PDGF-BB to prevent PDGF-BB binding to PDGFβ receptor.

Acknowledgements

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I thank my supervisor Dr. Michael Beazely for his continuous academic guidance and academic counselling, his patience in supporting the various stages of my project and for giving me the opportunity to conduct this research in his laboratory. I would also thank my committee advisors, Dr. Roderick Slavcev and Dr. Shawn Wettig for their support and advice for the last two years.

I would also like to thank Lucy Liu for giving me the opportunity to continue her project regarding involvement of Aβ42 in PDGF-BB signalling as well as teaching me various techniques in cell culture. I would also like to thank my colleagues Maryam Vasefi, Jeff Kruk and Azita Kouchmeshky for their support, helping in lab maintenance and teaching me various techniques.

Dedication

I dedicate this thesis to my mother, Shaheen Afroz, for supporting me during the two years of this project and my father, Golam Moula, for providing me information regarding graduate studies which greatly helped me in my research.

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List of Abbreviations

α7nAChRs = α7-containing nicotinic acetylcholine receptors

Aβ = Beta-amyloid

ABAD = Aβ-binding alcohol dehydrogenase

AD = Alzheimer's disease

ADDLs = Aβ-derived diffusible ligands

AGEs = advanced glycation end products

AICD = APP intracellular domain

AMPA receptor = α-amino-3-hydroxy-5-methyl-4-isoxazole propionate receptor

AMY-3 = Amylin receptor

Aph-1 = anterior pharynx-defective 1

APP = amyloid precursor protein

BACE = β-APP cleaving enzyme

beta(2)AR = Beta (2) adrenergic receptor

cAMP = cyclic AMP

CNS = central nervous system

DMEM = Dulbecco's Modified Eagle Medium

DMSO = dimethyl sulfoxide

EDTA = Ethylenediaminetetraacetic acid

EGTA = ethylene glycol tetraacetic acid

ERK = Extracellular signal-regulated kinases

ER = endoplasmic reticulum

GSK3β = glycogen synthase kinase β

HBD = heparin-binding domain

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIF-1α = hypoxia inducible factor-1α

HNE = 4-hydroxynonenal

IGF1R = Insulin-like growth factor receptor type 1

JNK = c-Jun N-terminal kinases

LTD = long-term depression

LTP = long-term potentiation

MAPK = mitogen-activated protein kinase

NGF = nerve growth factor

NMDA receptor = N-methyl-D-aspartate receptor

$p75^{NTR}$ receptor = p75 neurotrophin receptor

PBS = phosphate buffered saline

PDGF = platelet-derived growth factor

PEN-2 = presenilin enhancer 2

PI3K = phosphoinositode 3-kinase

PKA = protein kinase A

PLCγ = phospholipase Cγ

PTEN = phosphatase and tensin homolog

RAGE = receptor for advanced glycation end products

ROS = reactive oxygen species

RTK = receptor tyrosine kinase

SDS PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

TGF = Transforming growth factor

TrkA = tyrosine kinase receptor A

Chapter 1: Introduction

1.1 History of Alzheimer`s disease:

Dr. Alois Alzheimer in 1906, while performing post-mortem examinations of the brain of an individual with dementia, identified neuropathological features including neurofibrillary tangles and neuritic plaques[1]. This dementia was later named Alzheimer's disease (AD). Almost 80 years later (1984), Glenner and Wong isolated a small peptide, beta-amyloid (Aβ), from neuritic plaques of brains afflicted with AD[2].

1.2 Alzheimer`s disease pathology:

Alzheimer's disease is a late-onset neurological disorder resulting in neuronal dysfunction, a decline of cognitive and memory capabilities, and ultimately death. It affects 5.4 million Americans and is believed to cost $385 billion per year[3, 4]. It is characterized by extracellular aggregates of Aβ peptide in the brain and neurofibrillary tangles of paired helical filaments composed of hyperphosphorylated tau protein. The Aβ peptide is produced from processing of the amyloid precursor protein (APP), a type 1 integral membrane glycoprotein. In the amyloidogenic pathway, β-secretases and γ-secretases sequentially cleave APP to produce the pathogenic Aβ peptides[5]. β-secretase (β-APP cleaving enzyme (BACE)) is a type 1 transmembrane aspartyl protease[6] and γ-secretase is a multicomponent complex composed of presenilin-1/presenilin-2, (anterior pharynx-defective 1) Aph-1, nicastrin, PEN-2 (presenilin enhancer 2)[7]. Cleavage of APP by β-secretase at the ectodomain results in the production of a soluble APP-β domain and membrane associated APP C-terminal fragment C99 which is 99 amino acids long[8]. Then γ-secretase cleaves the C99 fragment to release the C-terminus of APP

known as APP intracellular domain (AICD) and Aβ peptides which spontaneously aggregate form fibrils, ultimately producing the plaques in the brain initially observed by Dr. Alzheimer (Figure 1).

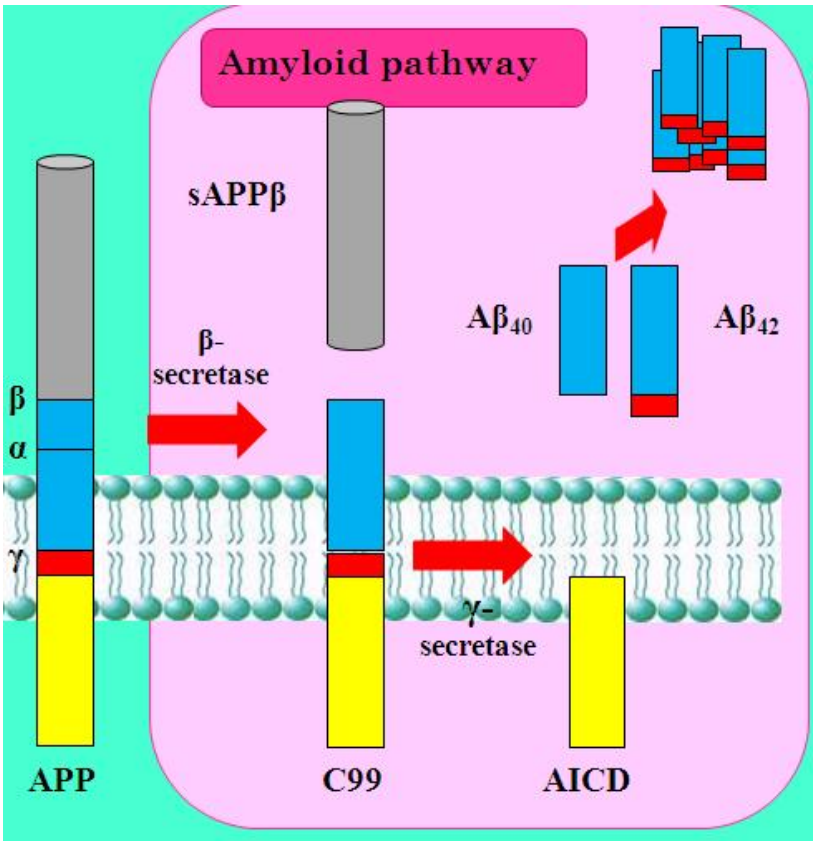


Figure 1: APP cleavage to Aβ. The type 1 integral membrane glycoprotein APP is cleaved by β-secretase to produce soluble APP-β domain (sAPPβ) and membrane associated APP C-terminal fragment C99. C99 is cleaved by γ-secretase to produce APP intracellular domain (AICD) and Aβ peptides[9].

Aβ peptides are between 38-43 amino acids in length and the majority of the peptides are 40 or 42 amino acids long[10, 11]. Aβ42 (and Aβ43) peptides are more neurotoxic than Aβ40. For example, mutations in the Aβ C-terminal of APP that increase the Aβ42/Aβ40 ratio are sufficient

to cause early onset Alzheimer's[12, 13]. This suggests that the ratio of Aβ42/Aβ40 may be more important than the absolute quantity of Aβ40 and Aβ42. Aβ42 is more hydrophobic and more fibrillogenic and is involved in the assembly of Aβ into higher order structures, from dimers to insoluble plaques that are found as deposits in the brain[14]. The difference in amino acid sequence between Aβ40 and Aβ42 is found in Figure 2. In addition to ~ 40 residue Aβ, the 11-residue Aβ fragment representing amino acid sequences 25-35 of full length Aβ (Aβ25-35) is used in various studies because it is small, possesses all the characteristics including: 1) neurotoxic, 2) neuroprotective, 3) amphilic, 4) aggregation characteristics of the full length Aβ peptide[15].

Peptide	Sequence
Aβ(1-42)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Aβ(1-40)	----------

Figure 2: Amino acid sequence of Aβ40 and Aβ42. The difference in amino acid sequence between Aβ40 and Aβ42 is shown[16].

Tau is a microtubule-associated protein (MAP) which is involved in the stability and assembly of microtubules. Microtubules are necessary for axoplasmic flow which is critical for neuronal function. The activity of the neuronal protein tau is regulated by its degree of phosphorylation: tau needs 2-3 mol of phosphate/mol of protein as a level of phosphorylation for its optimal activity. Hyperphosphorylation of tau, as observed in AD, results in decrease of microtubule assembly activity and binding of tau to microtubules. Hyperphosphorylation of tau during AD results in intraneuronal tangles of paired helical filaments (PHFs) which are correlated with dementia. Hyperphosphorylated tau in AD is found as; 1) polymerized into neurofibrillary tangles of PHF mixed with straight filaments (SF) and 2) non-fibrillized form in

the cytosol. Interestingly, tau polymerized as neurofibrillary tangles is inert whereas cytosolic hyerphosphorylated tau (P-tau) which may represent up to ~40% of total abnormal tau does not interact with tubulin/microtubules and sequesters normal tau, MAP1A/MAP1B and MAP2, resulting in inhibition and disassembly of microtubules[17-19]. Therefore, the toxic AD P-tau is involved in disassembly and inhibition of microtubule activity which is detrimental to microtubule-dependent neuronal activity[17-19]. It has been thought that production and/or deposition of Aβ precedes neurofibrillary tangle formation. However, studies suggest that tau is involved in mediating Aβ toxicity. For example, tau knock-out neurons are resistant to Aβ-neurotoxicity and cognitive impairment were shown to be rescued upon crossing a APP transgenic mice with a tau knock-out line[20].

Aβ peptides spontaneously aggregate in solution and it is thought that small oligomers of Aβ exert their neurotoxic effects: the promotion of tau hyperphosphorylation, neuronal dysfunction and neuronal loss[14]. Oxidative stress can increase the production of Aβ and at the same time can worsen cell membrane damage, cytoskeleton alteration, and cell death. Reaction between ROS and Aβ produce phenoxy radicals of Aβ at Tyr10 and this result in increased neurotoxicity and further acceleration of Aβ aggregation. Increases in the level of lipid peroxidation in the brains of AD patients have also been observed. Lipids in cell membranes undergoing oxidative modification results in structurally damaged membranes and production of aldehydic end products such as 4-hydroxynonenal (HNE) which themselves are oxidative and can impair cellular function[21, 22]. Toxic forms of aggregated Aβ can also result in Ca^{2+} influx into neurons by inducing membrane associated oxidative stress, which results in the neurons being more vulnerable to excitotoxicity and apoptosis[23]. In addition, Aβ can bind to mitochondrial Aβ-

binding alcohol dehydrogenase (ABAD) which is a mitochondrial protein that is up-regulated in the temporal lobe of AD patients. This interaction increased ROS production and cell death and caused spatial and learning impairments in 5-month old AβPP/ABAD double transgenic mice[24]. These molecular mechanisms result in cell death, and present as cognitive impairment and memory deficits at the macro level[25].

Glutamate-mediated excessive stimulation of N-methyl-D-aspartate (NMDA) receptors and α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) receptors regulate synaptic activity and NMDA receptor activation results in calcium influx, and if excessive, causes free radical generation, mitochondrial dysfunction, and additional intracellular cascades which lead to neuronal death, termed excitotoxicity. NMDA receptors are overactivated by excess glutamate released in AD in a tonic manner and this is coupled in a "feed-forward" pathway that results in additional Aβ production. Extended activation of extrasynaptic NMDA receptors increase β-secretase mediated cleavage of APP leading to increased Aβ production. Aβ can increase Ca^{2++} influx through various mechanisms which make the neurons vulnerable to excitotoxicity. For example, Aβ and NMDA receptors can induce endoplasmic reticulum (ER) stress which alters calcium homeostasis. In addition, Aβ oligomers can induce NMDA receptor activation leading to cellular damage and excitotoxicity. Furthermore, Aβ can reduce glutamate uptake at the synaptic cleft leading to increased glutamate levels. The increase in glutamate levels would increase NMDA receptor activation, and the feed-forward relationship between Aβ and NMDA/glutamate continues[26].

Patients with cerebral infarction and stroke have higher risks of developing AD. Severe and extended periods of hypoxia can lead to neuronal loss and memory impairment. Hypoxia can up-regulate expression of BACE1 gene resulting in higher levels of β secretase activity. In addition, hypoxia increases Aβ production, neuritic plaque formation, and memory deficits. Hypoxia induces the expression of hypoxia inducible factor (HIF)-1α which binds to the promoter of APH-1 to up-regulate γ-secretase expression since APH-1 is a subunit of γ-secretase. These evidences indicate that hypoxia increases β- and γ-secretase activities which accelerate APP cleavage to increase Aβ production and plaque formation[27-29]. In addition, analysis of post-mortem samples of human AD brain revealed that APP levels are increased after mild and severe brain ischemia[30].

Hyperglycemia which is involved in diabetes mellitus increases the risk of AD. Hyperglycemia increases the production of advanced glycation end products (AGEs) which are senescent protein derivatives formed from the auto-oxidation of glucose and fructose. The link between AGEs and AD is that tau and Aβ are substrates for glycation. Increase of extracellular AGEs formation occurs in amyloid plaques in different cortical areas and it was speculated of the involvement of AGEs in Aβ conversion from monomers to oligomers. AGEs can interact with the receptor for advanced glycation end products (RAGE) to stimulate oxidative stress. In a study conducted by Kim, B. et al., glucose treatment of rat embryonic cortical neurons resulted in apoptosis, caspase-3 activation, tau cleavage and these effects were increased when co-treated with Aβ. In addition, the authors concluded that hyperglycemia is one of the major factors that induce tau phosphorylation *in vitro* and *in vivo*[31, 22].

1.3 PDGF as a neurotrophic factor

Taken together, there is substantial amount of evidence suggesting that ischemia, NMDA receptor overactivation, and ROS production promote or worsen AD. The platelet-derived growth factor (PDGF) is a mitogenic growth factor crucial to mammalian development. It contains two disulfide-bonded polypeptide chains, A and B, isoforms dimerize to bind α and β PDGF receptors. PDGFβ receptors are primarily activated by the PDGF-BB ligand dimer, resulting in receptor dimerization and subsequent receptor autophosphorylation and kinase activation[32]. PDGF-BB treatment of primary rat cell culture results in neurite outgrowth and prolonged survival[33, 34]. In addition, PC12 neuronal cell differentiation is mediated by PDGFβ receptors, indicative of the role of this system in neuronal differentiation[32].

PDGF receptors (PDGFRs) are involved in activating various signalling pathways with numerous cellular beneficial outcomes. PDGFRs through the Grb2 and Shc activate the Ras-MAPK pathway which leads to activation of Raf-1 and MAPK cascade[32]. MAPK signalling is involved in stimulating gene expression that leads to cell growth, migration and differentiation. In addition, PDGFRs can activate PI3K resulting in actin reorganization, directed cell movement, inhibition of apoptosis and cell growth[32]. Furthermore, PDGFRs can bind and activate PLCγ which stimulates cell growth and motility[32]. PDGFRs can activate integrins resulting in cell proliferation, migration and survival[35].

PDGF has been shown to be neuroprotective against oxidative stress and glucose deprivation[36, 37]. Other neuroprotective actions of PDGF include protection against glutamate/NMDA induced N-methyl-D-aspartate (NMDA) receptor excitoxicity. This

neuroprotection is through preferentially inhibiting NR2B-containing NMDA receptors, decreasing surface expression of NR2B subunits, and altering their phosphorylation level (Figure 3)[38, 39]. The brain itself may use PDGF signalling as an endogenous neuroprotective system: after ischemic events *in vivo* the expression of PDGF-B chain mRNA and PDGFβ receptor protein levels rise and exert neuroprotective effects[40, 41, 42]. PDGF also has neuroprotective effects against HIV-1 Tat-induced neuronal apoptosis, possibly by decreasing extracellular glutamate levels, both of which lead to HIV-associated neurological diseases, including dementia[43]. Thus, in addition to being a growth factor, PDGF also promotes growth and survival of neurons; therefore it could also be described as a neurotrophic factor. Thus, promoting the activity of PDGF signalling has been demonstrated to be protective in models of neurodegenerative diseases both *in vitro* and *in vivo*. These lines of research resulted in the Beazely lab pursuing the possibility that the application of PDGF-BB might be neuroprotective against Aβ-induced neuronal cell death.

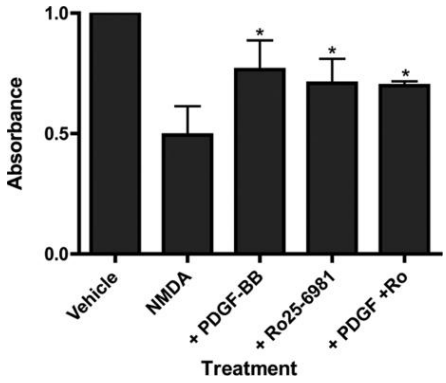


Figure 3: PDGF-BB is neuroprotective against NMDA toxicity. Cultured hippocampal neurons were pretreated for 10 min with vehicle, 10 ng/ml PDGF-BB, 2.5 μM Ro25-6981 (NR2B antagonist), or both after which they have been incubated with vehicle or 100 μM NMDA, 1 μM glycine for 3 min. After 24 hrs, cell viability was determined by enzyme-linked immunosorbent assay. (n=4, * = $p < 0.05$ compared with NMDA-treated cells)[39].

1.4 PDGF and Alzheimer's disease.

Previously, Lucy Liu in the Beazely lab investigated whether PDGF-BB application to primary hippocampal neurons would be neuroprotective against Aβ42. Despite being neuroprotective against several neuronal insults, PDGF-BB failed to reverse Aβ42 toxicity and Aβ42 5μM was shown to maximally reduce cell viability in the absence or presence of PDGF-BB for SH-SY5Y cells and primary hippocampal neurons. Preliminary evidence was also collected suggesting that Aβ42 oligomers might impair PDGF-BB-induced PDGFβ receptor phosphorylation. Interestingly, similar to what is observed after cerebral ischemia, PDGF-BB is found in higher levels in patients of AD[44]. The PDGF system is also involved in APP processing. PDGFβ receptor activation by PDGF-BB induces cleavage of APP, which requires γ-secretase activity. PDGF receptor activation also leads to activation of non-receptor tyrosine kinase Src which increases Aβ production[45]. Thus, PDGF-BB can mediate Aβ production through Src activation.

1.5 The role of Aβ interactions with neuronal receptors Alzheimer's disease.

There is a growing body of evidence suggesting that the neurotoxic capabilities of Aβ-derived diffusible ligands (ADDLs), i.e. Aβ oligomers, are due to their binding to neuronal cell surface receptors and membranes. These interactions may lead to synaptic dysfunction via impaired LTP or LTD facilitation and may negatively affect memory and cognition before cell death. Significant amount of evidence relates to the involvement of membrane proteins for Aβ binding to neuronal cell surfaces[46-49]. Examples of interaction between Aβ and receptors/ligands as well as the pathogenic consequences of such interactions are listed below in Table 1.

Table 1: impact of interaction of beta amyloid with various receptor/ligands

Protein interaction	Phenotypic effect	Form of Aβ	Cell type
Amylin receptor (AMY-3)[51]	increase cytosolic cAMP, Ca(2+), cell death[50]	Aβ42 oligomer	HEK293
VEGFR-2 (vascular Endothelial growth Factor receptor 2)[52]	prevents binding of VEGF, inhibit migration of endothelial cells, anti-angiogenesis	Aβ42 dimer	Endothelial cells
p75 neurotrophin receptor (p75NTR)[53]	neuronal cell death	Aβ42	F11 neuronal hybrid cells
Transforming growth Factor-beta (TGF)[54]	Enhanced Aβ oligomerization, neurotoxicity	Aβ40 peptide	PC12 cells
Beta (2) adrenergic Receptor (beta(2)AR)[55]	induces PKA dependent AMPA receptor hyperactivity causing Ca (2+) influx and excitoxic neuronal cell death	Aβ42 dimer	FC/HEK293 cells
α7-containing nicotinic acetylcholine receptors (α7nAChRs)	neuronal cell death[56]	Aβ42 (12-28)	Human Neuroblastoma cells

1.6 Hypothesis, methodology and project outline

Based on previous research findings, we hypothesized that Aβ42 inhibits PDGF-BB-induced PDGFβ receptor phosphorylation (and downstream signalling pathways) by physically interacting with the PDGFβ receptor. We anticipated that this inhibition would be competitive and dose-dependent. To determine the effects of Aβ42 on effectors downstream of the PDGFβ receptor we chose to examine Akt activation (using phosphorylation at Ser473 as a readout)[57]. The primary method used was western blotting with anti-phosphotyrosine antibodies and immunoprecipitation experiments. Please refer to materials and methods sections for more detail about the protocols.

Chapter 2: Results

2.1. Aβ inhibits PDGF-BB signalling

2.1.1 Aβ42 inhibits PDGF-BB-induced PDGFβ receptor phosphorylation at site Tyr1021 in SH-SY5Y cells

Previous work in the Beazely lab by Lucy Liu suggested that Aβ42 oligomers were able to inhibit the activation of the PDGFβ receptor by its primary ligand, PDGF-BB. Therefore, this finding was validated by repeating the experiment with the same treatments under the same conditions using the same cell line SH-SY5Y cells, which is outlined in Figure 4.

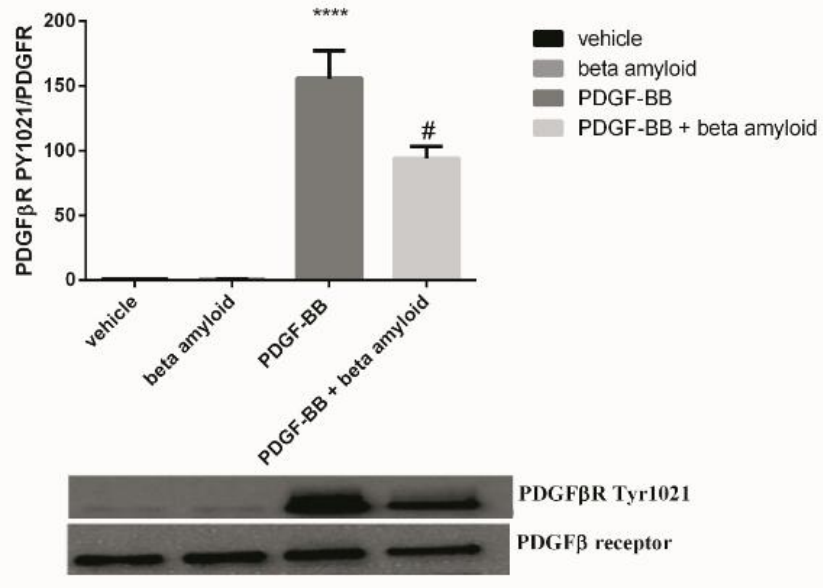
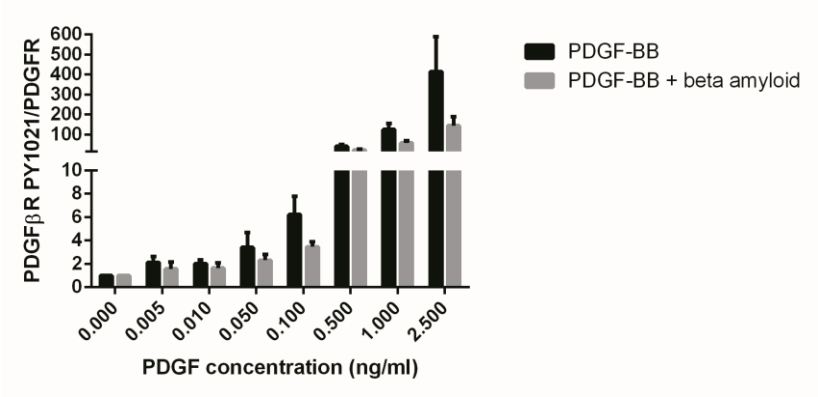


Figure 4: Aβ42 inhibits PDGF β receptor Tyrosine 1021 phosphorylation in presence of PDGF-BB in SH-SY5Y cells: SH-SY5Y cells were treated with vehicle, Aβ42 oligomer 5 μM for 10 min, PDGF-BB 1 ng/ml for 5 min, or Aβ42 oligomer for 10 min followed by PDGF-BB 1 ng/ml for 5 min. (n = 3). Representative Western blots analyzed using a PDGFβ receptor Tyr1021 antibody followed by a PDGF β receptor antibody are shown. (*= $p<0.05$ compared to vehicle treated cells, #= $p<0.05$ compared to PDGF-BB treated cells)

2.1.2 Increasing the concentration of PDGF-BB is not able to overcome the inhibition by Aβ42 oligomers

SH-SY5Y cells were exposed to multiple concentrations of PDGF-BB for 5 min in presence or absence of Aβ42 oligomers to evaluate whether Aβ42 inhibits PDGF-BB-induced PDGFβ receptor Tyr 1021 phosphorylation across multiple concentrations of PDGF-BB treatment (Figure 5). The results show that Aβ42 was able to inhibit PDGFβ receptor Tyr1021 phosphorylation across multiple concentrations of PDGF-BB and that even at high concentrations Aβ42 oligomers are still able to inhibit the activation of the PDGFβ receptor by PDGF-BB, further validating the absence of PDGF-BB induced PDGFβ receptor phosphorylation effects in presence of Aβ42.

A.



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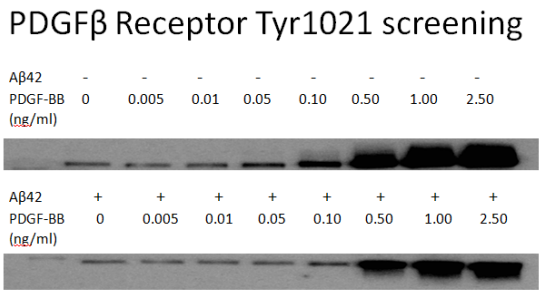

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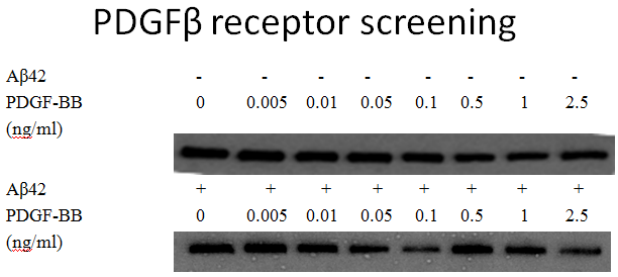


Figure 5: Aβ42 inhibits PDGFβ Receptor Tyr1021 phosphorylation in presence of PDGF-BB in SH-SY5Y cells at all concentrations of PDGF-BB tested. SH-SY5Y cells were exposed to increasing concentrations of PDGF-BB ranging from 0-2.5ng/ml for 5 min in presence and absence of 5 μM Aβ42 oligomer 10min. (A) Expression levels of PDGFβ receptor Tyr1021 was normalized to PDGFβ receptor for different treatments of PDGF-BB in presence/absence of Aβ42 (n=5-7). Representative western blots analyzed using a (B) PDGFβ receptor Tyr1021 antibody and (C) PDGFβ receptor is shown.

2.1.3 Aβ42 oligomers also inhibit PDGF-BB induced PDGFβ receptor phosphorylation at other tyrosine residues

In order to evaluate whether Aβ42 oligomers inhibit PDGF-BB-induced PDGFβ receptor phosphorylation at other tyrosine residues, we analyzed treated lysates with additional antibodies against tyrosines 740, 751, 771 (Figure 6). Aβ42 oligomers do indeed inhibit the phosphorylation of these tyrosine residues, in some cases to a greater extent to what we observed at tyrosine 1021.

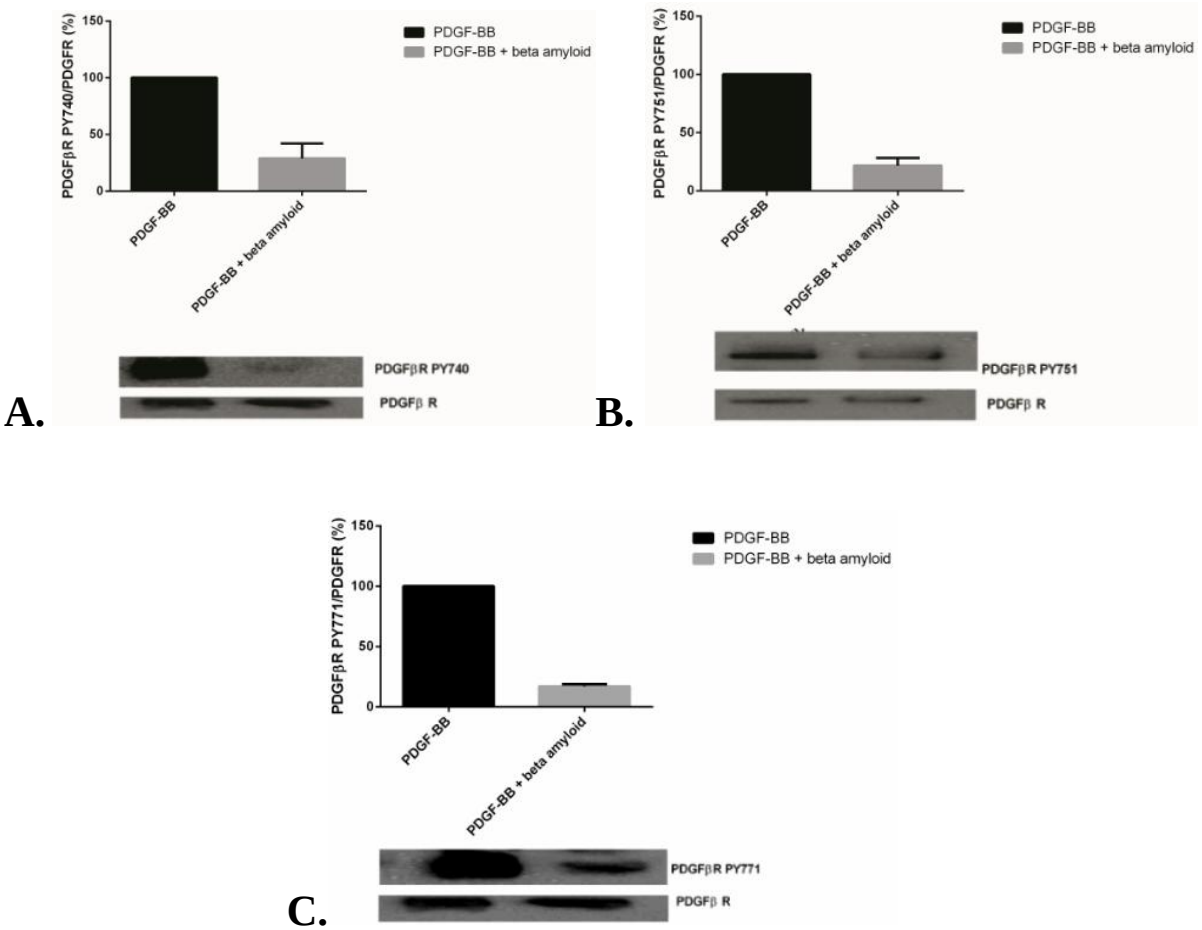


Figure 6: Aβ42 inhibits multiple PDGFβ receptor tyrosine phosphorylation sites in presence of PDGF-BB. Briefly, SH-SY5Y cells were treated with PDGF-BB 1ng/ml 5 min, Aβ42 oligomer 10 min + PDGF-BB 1ng/ml 5 min. Western blot was performed and all the membranes were incubated with PDGFβ receptor antibody. The membranes were stripped and rescreened with (A) PDGFβ receptor Tyr740 antibody (n=3), (B) PDGFβ receptor Tyr751 antibody (n=3), (C) PDGFβ receptor Tyr771 antibody (n=3). Data shown is the fold inhibition of PDGF-BB-induced phosphorylation by Aβ42 oligomers. Data is (A) statistically significant for PDGFβ receptor Tyr740 phosphorylation inhibition ($p<0.05$), (B) statistically significant for PDGFβ receptor Tyr751 phosphorylation inhibition ($p<0.05$), (C) statistically significant for PDGFβ receptor Tyr771 phosphorylation inhibition ($p<0.05$).

2.2 Aβ42 inhibits effectors downstream of the PDGFβ receptor.

To determine if Aβ42 oligomers also inhibit the activation of effector proteins downstream of the PDGFβ receptor, we treated SH-SY5Y cells with PDGF-BB in the absence or presence of Aβ42. We first examined the phosphorylation of Akt at serine 473. The PDGFβ receptor is a tyrosine kinase and a component of the receptor intracellular signalling machinery is the PI3K (phosphoinositode 3-kinase)/AKT/mTOR pathway which is involved in regulating cell proliferation and cell survival[58]. Therefore, evaluating AKT phosphorylation at site serine 473 is an appropriate assessment of the downstream consequences of Aβ42 mediated inhibition of PDGF-BB-induced receptor phosphorylation. Similar to the results with PDGFβ receptor phosphorylation sites, Aβ42 reduced the activation of Akt across all concentrations of PDGF-BB treatment. This suggests that the observed changes in PDGFβ receptor phosphorylation by Aβ42 results in changes to the activation state of downstream effectors.

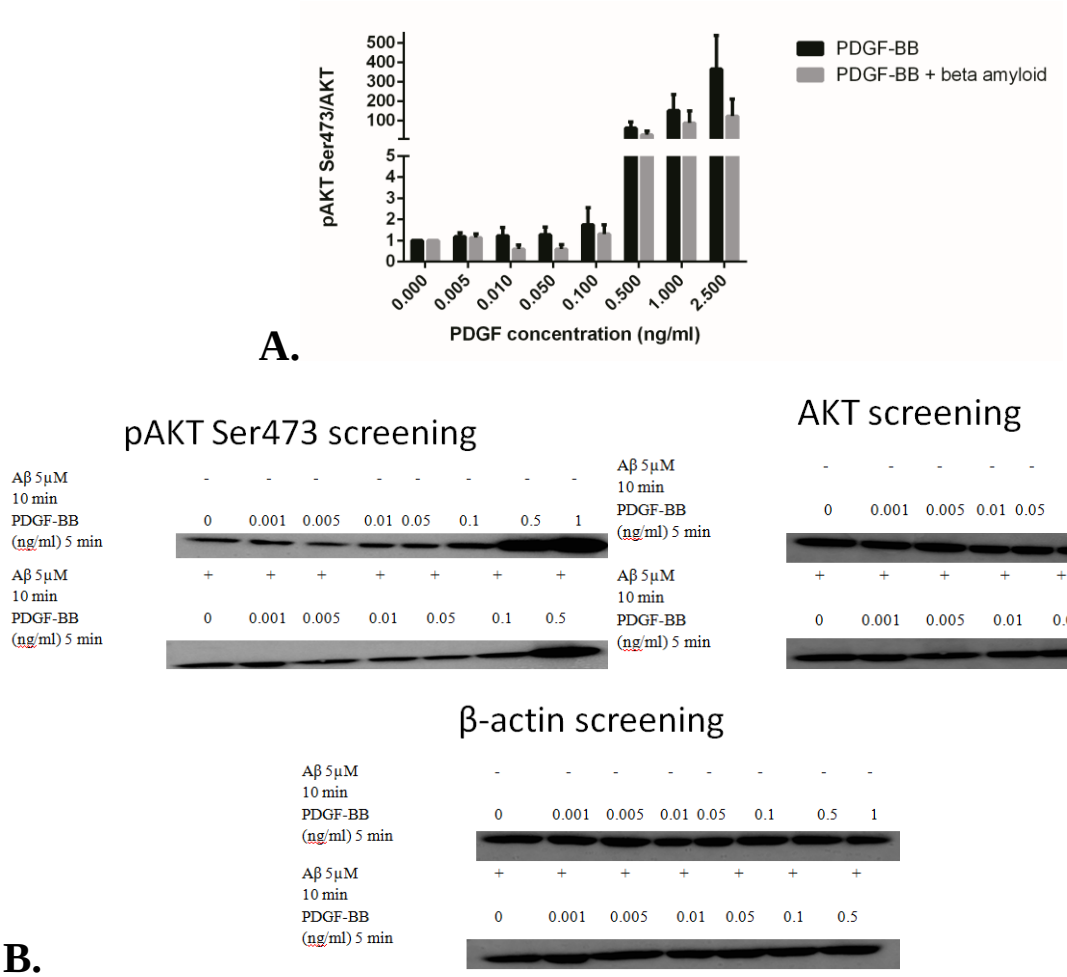


Figure 7: Aβ42 inhibits pAKT Ser473 phosphorylation in the presence of PDGF-BB in SH-SY5Y cells. SH-SY5Y cells were exposed to various concentrations of PDGF-BB (0-2.5ng/ml) for 5 min in presence and absence of 5μM Aβ42 oligomer for 10 min. Western blot was performed. Membranes were screened with pAKT Ser473 antibody, stripped, rescreened with AKT antibody, stripped and rescreened with β-actin antibody. (A) AKT expression was normalized to β-actin expression and pAKT Ser473 expression was normalized to the AKT/β-actin normalized values. (n = 3-6). (B) illustrates western blot membranes of pAKT Ser473, AKT and β-actin expression respectively. Often different experiments were conducted with differing concentrations of PDGF-BB treatment in presence and absence of Aβ42. However, the total number of independent experiments is 3-6 as indicated in part (A).

2.3 Mechanism of Aβ inhibition of PDGF-BB signalling

2.3.1 Aβ42 oligomers do not physically interact with the PDGFβ receptor

To evaluate whether or not the mechanism of PDGFβ receptor phosphorylation inhibition by Aβ42 oligomer was due to Aβ42 binding to PDGFβ receptor, an immunoprecipitation experiment was carried out. SH-SY5Y cells were incubated with Aβ42 oligomer 5μM for 10 min after which the cells were lysed and the protein lysates were exposed to PDGFβ receptor antibody in order to immunoprecipitate PDGFβ receptor protein from the lysate. After isolation of PDGFβ receptor, samples were analyzed by Western blotting to determine whether the PDGFβ receptor was pulled down and if so, whether Aβ42 was co-immunoprecipitated. The results in Figure 8 show that PDGFβ receptor did not bind to Aβ42 oligomer (6kDA) which suggests that the method of PDGFβ receptor phosphorylation inhibition in presence of ABβ42 and PDGF-BB involves a mechanism independent of Aβ42 oligomer binding to PDGFβ receptor.

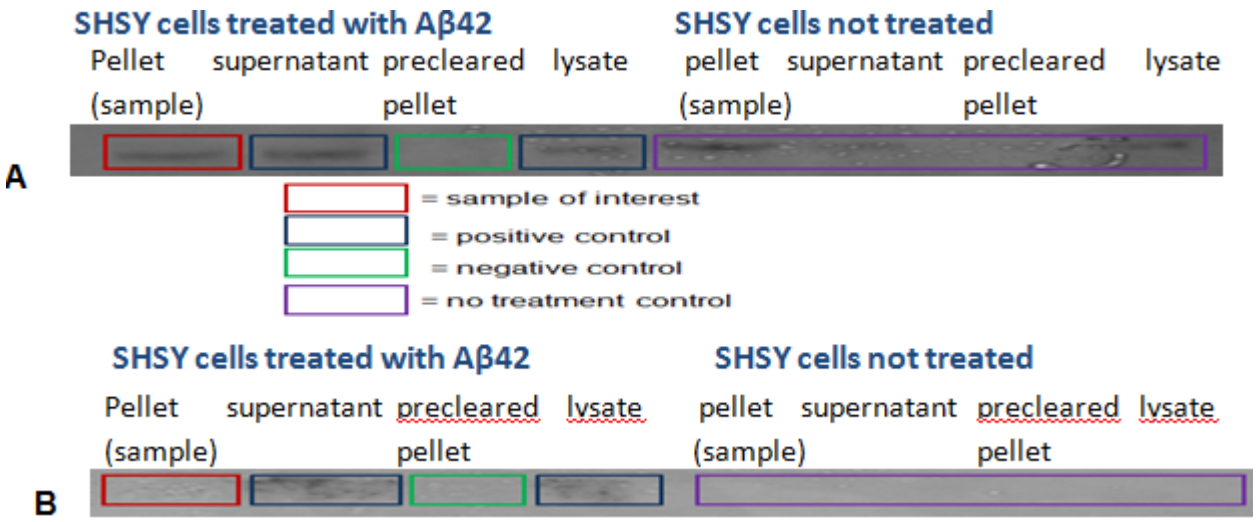
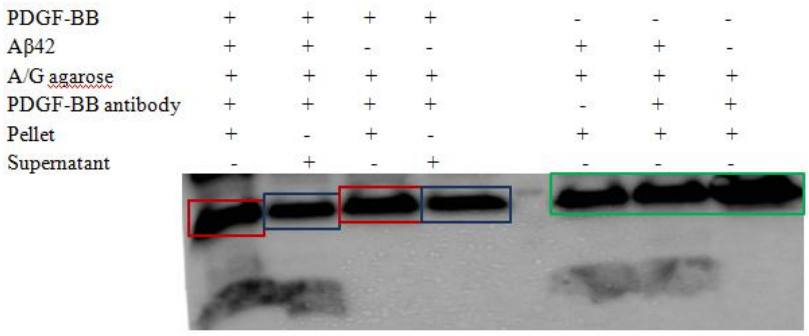


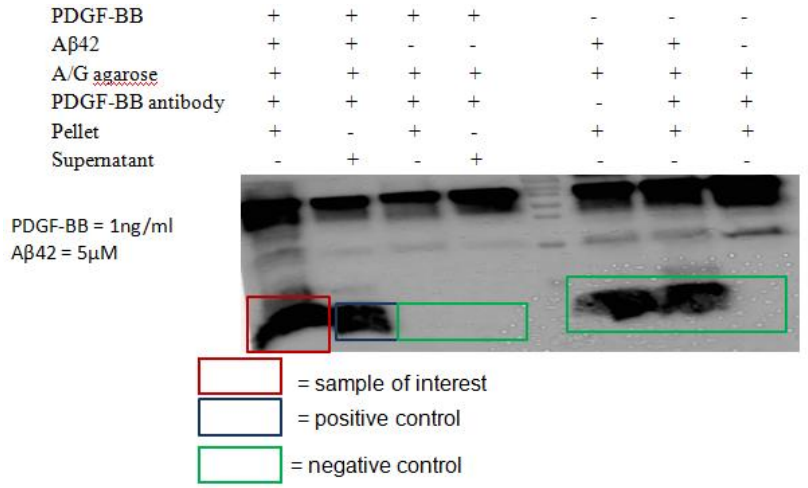
Figure 8: Aβ42 does not bind to PDGFβ receptor in SH-SY5Y cells. Briefly, SH-SY5Y cells were incubated in the absence or presence of Aβ42 (5μM) for 10 min after which cells were lysed, exposed to PDGFβ receptor antibody. The samples derived during the process of immunoprecipitation, 1) lysate, 2) precleared pellet, 3) supernatant, 4) pellet were screened through western blot to detect (A) for the presence of PDGFβ receptor (180 kDA) in the samples and (B) if Aβ42 (6 kDA) bound to the PDGFβ receptor that was immunoprecipitated (n = 3).

2.3.2 Aβ42 oligomers may bind to the PDGF-BB ligand

To identify whether the inhibition of PDGF-BB-induced PDGFβ receptor phosphorylation was inhibited by Aβ42 due to Aβ42 oligomer binding to PDGF-BB to prevent ligand binding to the PDGFβ receptor, immunoprecipitation experiment was carried out *in vitro*. This immunoprecipitation experiment differed from the previous immunoprecipitation experiment since proteins were not extracted from cells. Rather, protein solutions generated from stock, PDGF-BB at 1ng/ml and Aβ42 at 5μM were used to evaluate possible interactions between PDGF-BB and Aβ42. (This modification is further explained in the materials and methods "immunoprecipitation" section.)



A.



B.

Figure 9: *In vitro* immunoprecipitation experiment to test for interaction between PDGF-BB and Aβ42 oligomer. Briefly, Aβ42 5μM oligomer + PDGF-BB 1ng/ml + PDGF-BB antibody, PDGF-BB 1ng/ml + PDGF-BB antibody, Aβ42 5μM + PDGF-BB antibody were incubated overnight after which they were incubated with A/G agarose overnight. A/G agarose + PDGF-BB antibody were incubated overnight. A/G agarose + Aβ42 5μM oligomer were incubated 90mins to overnight. Western blot was performed on the supernatant and pellet samples obtained and the membranes were screened with (A) PDGF-BB antibody (n=3) and (B) Aβ42 antibody (n=3) to evaluate presence of PDGF-BB (14-18kDA in reduced form) in samples expected to have PDGF-BB in (A) and possible interactions between PDGF-BB and Aβ42 (6kDA) in (B).

A band in the 6kDA range in Figure 9B for the pellet sample of PDGF-BB 1ng/ml + Aβ42 5μM + PDGF-BB antibody + A/G agarose indicates a possible interaction between Aβ42 oligomer and PDGF-BB. However, in the pellet sample of A/G agarose + Aβ42 there was also a band in the size range of 6kDA. This indicates that Aβ42 oligomer might have been interacting directly with the A/G agarose IP beads. Since this negative control failed to work, we cannot make any claims about a potential PDGF-BB-Aβ42 interaction. In order to further evaluate for possible interactions between PDGF-BB and Aβ42, a reverse immunoprecipitation experiment was carried out which followed the same methodology as the *in vitro* experiment mentioned above but the IP was conducted using the Aβ42 antibody instead of PDGF-BB antibody to pull down Aβ42 to evaluate interactions between Aβ42 and PDGF-BB by screening western blot membranes with PDGF-BB antibody. This reverse immunoprecipitation experiment was carried out since if Aβ42 is binding to the A/G agarose beads, then pulling down Aβ42 with Aβ42 antibody is hypothesized to prevent interactions between Aβ42 and A/G agarose beads since Aβ42 would be binding to the antibody.

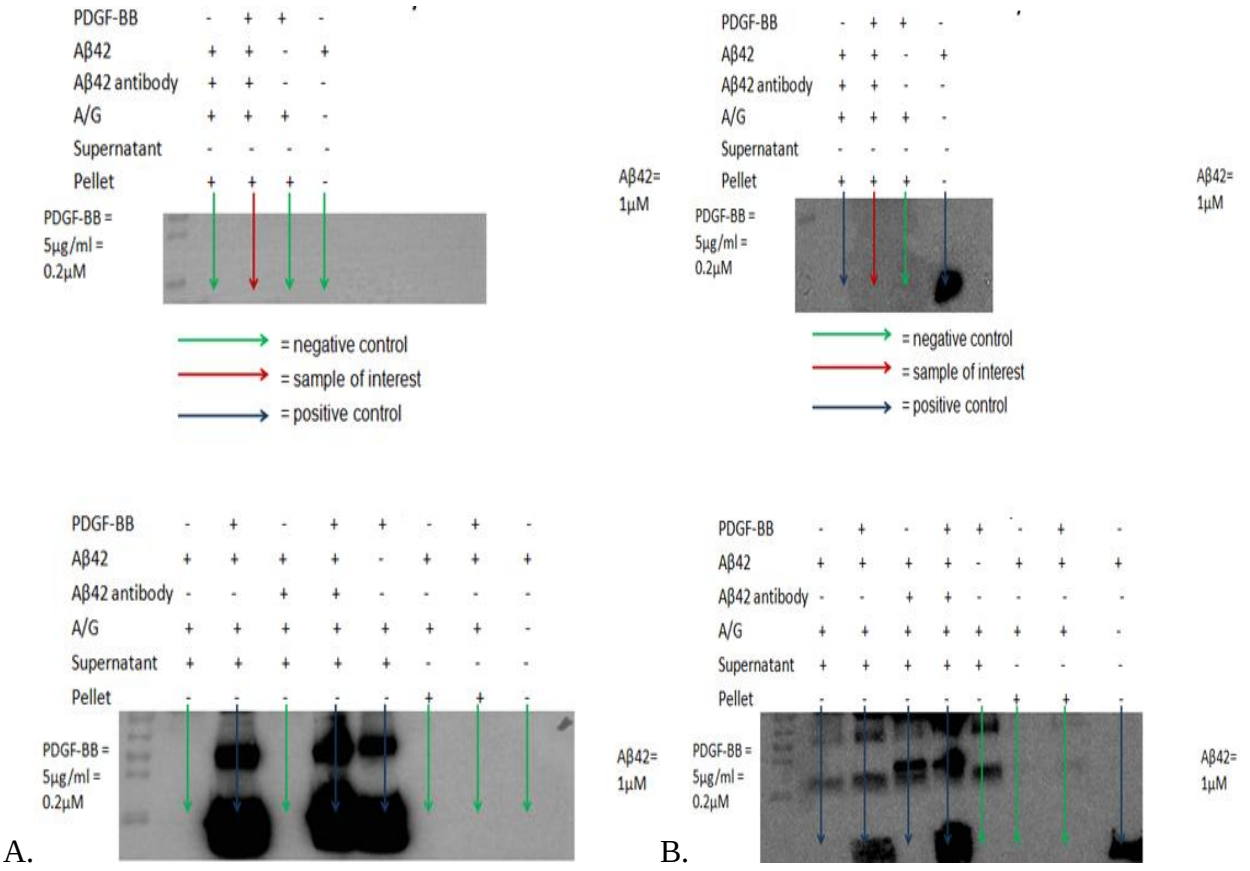


Figure 10: Reverse immunoprecipitation experiment to evaluate interactions between Aβ42 and PDGF-BB. Briefly, PDGF-BB (5µg/ml= 0.2µM) + Aβ42 1µM + Aβ42 antibody, Aβ42 1µM + Aβ42 antibody were incubated overnight. These samples and the following samples: Aβ42 1µM, Aβ42 1µM + PDGF-BB (5µg/ml= 0.2µM), PDGF-BB (5µg/ml= 0.2µM) were all incubated with A/G agarose for 90 to 150 mins. The supernatant and pellet samples were obtained and western blot was performed (n=2). The membranes were screened with (A) PDGF-BB antibody and (B) Aβ42 antibody to (A) detect for interactions between PDGF-BB (14-18kDA) and Aβ42 (6kDA) and (B) detect for presence of Aβ42 (6kDA) in supernatant samples containing Aβ42. The control was Aβ42 0.1µg to validate the efficiency of Aβ42 antibody.

During the *in vitro* reverse immunoprecipitation experiment, it was observed from Figure 10B for the pellet sample in lane 2 of top membrane and the pellet sample in lane 1 of top membrane that the Aβ42 antibody failed to pull down Aβ42 due to absence of band within the size range of Aβ42 (6kDA). Therefore, the absence of a band within the size range of PDGF-BB in Figure 10A lane 2 of top membrane pellet sample could be due to failure of Aβ42 antibody being unable to pull down Aβ42. Moreover, in Figure 10B there was no Aβ42 (6kDA) detected in any of the pellet samples including Aβ42 + A/G agarose, Aβ42 + A/G agarose + PDGF-BB indicating the absence of interaction between Aβ42 with A/G agarose. The absence of such interaction might be due to low concentrations of Aβ42 used (1μM) in comparison to before where 5μm Aβ42 was used. Some reasons as to why the immunoprecipitation experiment could have failed is due to lack of a appropriate antibody to pull down Aβ42. According to a study conducted by Patel, N. S. *et al*. which followed a similar immunoprecipitation protocol as our lab, the Aβ42 antibody 6E10 antibody was used. This antibody effectively pulled down Aβ42 from Aβ42 treated human umbilical vein endothelial cells (HUVEC) and was used to show the interaction between Aβ42 and VEGF receptor type 2 (VEGFR-2)[52]. Therefore, 6E10 antibody should be used to immunoprecipitate Aβ42 to evaluate interactions between Aβ42 and PDGF-BB in the future.

Chapter 3: Discussion

Previous work in the lab suggested that Aβ42 was able to prevent activation of PDGFβ receptor by its ligand, PDGF-BB. This finding was confirmed (Figures 4-6): if cells were pre-incubated with Aβ42, the application of PDGF-BB failed to maximally active the PDGFβ receptor. This is a particularly interesting and relevant finding with respect to the pathology of Aβ in Alzheimer's disease. PDGF-BB is neuroprotective (*in vitro* and *in vivo*) against several CNS insults including oxidative stress, glucose deprivation, glutamate/NMDA receptor excitoxicity, ischemic events, and HIV-1 Tat-induced neuronal apoptosis[36-43]. Therefore, in addition to Aβ42's other neurotoxic effects, it is able to inhibit a receptor tyrosine kinase (RTK) crucial for neuroprotection from other neuronal insults.

3.1 Mechanism of Aβ42 inhibition of PDGF-BB signalling

We did not detect a physical interaction between Aβ42 and the PDGFβ receptor. With respect to a possible interaction between Aβ42 and PDGF-BB, the results were inconclusive. Aβ have been shown to interact with various ligands of receptors which will be summarized in Table 2. For example, Aβ42 has been shown to interact with vascular endothelial growth factor (VEGF) which is an RTK ligand for VEGF receptor type 2 (VEGFR-2). VEGF is a growth factor for stimulating angiogenesis. VEGF expression is increased in the cerebral vasculature and cerebrospinal fluid of AD patients. In a study conducted by Yang, S. P. *et al*., VEGF was found to co-localize with amyloid plaques in AD patients and bind strongly to Aβ42 and Aβ40 (the disassociation constant between VEGF and Aβ40 was 50pM and the binding affinity for VEGF and Aβ42 was similar). VEGF was shown to bind to pre-aggregated Aβ under acidic conditions

and VEGF disassociated from VEGF/Aβ complex at a rate of 1.5% after 3 days. Interestingly, the binding of Aβ to VEGF does not appear to have a significant effect on VEGF binding to its receptor or the mitogenic activity of VEGF. These results indicate that VEGF co-aggregate with Aβ and does not affect the rate of Aβ aggregation, then binds to pre-aggregated Aβ and is released slowly from Aβ/VEGF complex[59-61]. In another study conducted by Yang, S. P. *et al*., Aβ was found to interact differentially with different forms of VEGF. Aβ42 did not bind to $VEGF_{121}$ but bound to the heparin-binding domain (HBD) of $VEGF_{165}$. Moreover, Aβ42 bound with greater affinity to the C-terminal end of HBD compared to the N-terminal end. The amino acid region 26-35 of Aβ42 was necessary for binding to $VEGF_{165}$. VEGF and HBD can reduce Aβ42-induced cell death of PC12 cells and slow the aggregation of Aβ42. In addition, VEGF reduces Aβ42 mediated reactive oxygen species (ROS) production. These results suggest that $VEGF_{165}$ is neuroprotective against Aβ42 due to $VEGF_{165}$ mediated inhibition of Aβ aggregation and ROS formation since these mechanisms can lead to neuronal death[62]. However, in our studies the possible Aβ interaction with PDGF-BB could result in an inhibition of PDGF-BB signalling and inhibit downstream proteins such as Akt involved in proliferation and neuroprotection.

In addition, transforming growth factor β (TGFβ) have been shown to interact with Aβ40 and enhances Aβ40 oligomerization and Aβ40 mediated neurotoxicity[63]. Therefore, interactions between Aβ and ligands in AD can lead to neuroprotection or neurodegeneration and perhaps during the later stages of AD, Aβ interaction with ligands involved in neurodegeneration overcomes neuroprotective interactions such that neurodegenerative interactions become the cause of progression of AD pathology.

Table 2: Beta amyloid interaction with receptor ligands

Ligand	Phenotypic consequence	Form of beta amyloid	Cell type
VEGF	inhibition of Aβ42 cell death, ROS production, Aβ aggregation[62]	Aβ42	PC12 cells
TGFβ	Aβ40 oligomerization, Neurotoxicity[63]	Aβ40	PC12 cells

3.2 **Aβ modulation of tyrosine kinase receptors and AD**

Aβ42, in addition to inhibiting the tyrosine kinase PDGFβ receptor essential for neuroprotection, is also able to interfere with the signalling of other receptor tyrosine kinases and neurotrophic factor receptor. Aβ42 modulation of RTKs led to increase in $p75^{NTR}$ expression and $p75^{NTR}$ activity which is neurotoxic. Increases in $p75^{NTR}$ receptor are thought to be involved in facilitating neurodegeneration by interacting with various ligands and co-receptors. For example, $p75^{NTR}$ receptor interacts with Sortilin to facilitate apoptosis caused by pro-neurotrophins such as proBDNF, proNGF and proNT-3[64-67]. Further evidence relating to the neurotoxic potential of $p75^{NTR}$ receptor is that there is an increase in the level of pro-neurotrophins in Alzheimer's brain and pro-NGF isolated from human AD brains through interaction with $p75^{NTR}$ receptor was shown to induce apoptotic cell death of neuronal cell cultures[68-69]. Moreover, $p75^{NTR}$ receptor

expression has been shown to be up-regulated by Aβ *in vitro* in SH-SY5Y cells and primary human neurons and $p75^{NTR}$ receptor is involved in Aβ oligomer-induced neuritic dystrophy and neuronal cell death *in vivo* and *in vitro*.

Interestingly, Aβ42 does not always act as an antagonist in growth factor systems (in contrast to our findings). For example, nerve growth factor (NGF) is initially synthesized as a proneurotrophin that binds to $p75^{NTR}$ receptor, and upon maturation of NGF it binds to tyrosine kinase receptor A (TrkA)[70]. TrkA undergoes a NGF-independent phosphorylation when exogenous Aβ42 is applied and this increased phosphorylation promotes neuronal cell death. The $p75^{NTR}$ receptor and phospholipase Cγ (PLCγ) activation was also shown to be involved in TrkA mediated neuronal death[71]. Aβ42 mediated neuronal death through TrkA phosphorylation is yet another example of a case where the consequences of Aβ modulation of RTK activity are negative with respect to neuronal survival.

Aβ42 can also indirectly modulate RTK activity in neurons. In another study by Bulbarelli, A. *et al*., treatment of hippocampal neurons with Aβ25-35 lead to increase in apoptotic cell death and these toxic insults may activate neurotrophin signalling pathways. Upon further investigation the researchers determined that Aβ25-35 treatment resulted in a significant up-regulation of NGF and TrkA mRNA expression. Ultimately, an increased amount of NGF protein was released into the media that increased TrkA activity in an autocrine-dependent manner. Aβ25-35 resulted in an increase in Akt phosphorylation at site serine 473 as well as an increase in serine 9 phosphorylation of glycogen synthase kinase β (GSK3β). Interestingly, the

increase in Akt phosphorylation was measured prior to NGF release, indicating that Akt was not being activated by NGF activation of TrkA. These findings are relevant to Alzheimer's pathology because Akt is a serine-threonine kinase which phosphorylates and inactivates Gsk3β, a kinase thought to be involved in tau hyperphosphorylation[72-75].

Insulin-like growth factor receptor type 1 (IGF1R) is a RTK activated by ligands insulin-like growth factor 1 and 2 (IGF-1, 2). IGF1R is an example of another RTK whose phosphorylation is modulated by Aβ. IGF1R is involved in controlling $p75^{NTR}$ expression in SH-SY5Y cells and primary mouse neurons[76-77]. The correlation between IGF1R, Aβ and $p75^{NTR}$ receptor was further evaluated by Ito, S. *et al*. ADDLs (Aβ-derived diffusible ligands) are Aβ42 oligomers which induced $p75^{NTR}$ expression in SH-SY5Y human neuroblastoma cells through phosphorylation of IGF1R. *In vivo* microinjection of ADDLs in mice increased $p75^{NTR}$ expression by 1.4-fold in the ispsilateral hippocampus compared to contralateral hippocampus. Furthermore, microinjection of ADDLs in the mouse hippocampi increased IGF1R phosphorylation within 30 mins. Further examination of the hippocampi of 6-month old AβPPswe/PS1dE9 AD model mice with accumulated Aβ42 showed higher levels of IGF1R phosphorylation and $p75^{NTR}$ in comparison to age-matched wild-type mice. All of these findings indicate that Aβ42 stimulate IGF1R phosphorylation which leads to $p75^{NTR}$ expression in the hippocampus. Moreover, since $p75^{NTR}$ expression was found to be involved in Aβ-mediated neurodegeration and since $p75^{NTR}$ can stimulate Aβ production in neurons through ceramide-induced stabilization of β-site AβPP cleaving enzyme 1 (BACE1 or β-secretase), Aβ-mediated upregulation of $p75^{NTR}$ expression via IGF1R phosphorylation leads to neuronal cell death and Aβ production, thus accelerating the development of AD during the early stages. Furthermore,

Aβ toxicity is decreased due to decreased IGF1R signalling in IGF-1R$^{+/-}$, 2xTg AD model mice. Moreover, increasing IGF1R pathway activation in p44$^{+/+}$ transgenic mice increased p75^{NTR} expression in the brain which accelerated aging and shortened lifespan[78-80].

3.3 PDGF neuroprotection against excitotoxicity, hypoxia, oxidative stress in relation to AD

Excitotoxicity can occur via excess stimulation of NMDA receptors by glutamate. There is considerable evidence of the involvement of excitotoxicity in AD[81-85]. NMDA receptors are tetrameric consisting of two obligate NR1 and two variable NR2 subunits. NR2A and NR2B are the dominant NR2 forms in hippocampus. NR2A containing NMDA receptors are mostly synaptic whereas NR2B containing NMDA receptors are extrasynaptic[86-90]. According to a study conducted by Beazely, M. A. *et al*., PDGF-BB selectively inhibited NR2B- and not NR2A-containing NMDA receptor mediated currents in CA1 hippocampal neurons and facilitated long-term depression in NR2B dependent fashion. Moreover, PDGF-BB treatment of hippocampal neurons decreased surface expression of NR2B subunits and their level of phosphorylation. Thus, PDGF-BB prevents over-stimulation of NMDA receptor. Furthermore, PDGF-BB and PDGFβ receptors are up-regulated following neuronal injury which resulted in PDGFβ receptor activation that was found to be neuroprotective against glutamate-induced neuronal damage and this neuroprotection was occluded by NR2B antagonist Ro25-6981, suggesting the involvement of NR2B-containing NMDA receptor in PDGFβ receptor mediated neuroprotection[39]. Therefore the mechanism whereby PDGF-BB exerts its neuroprotective effects is through PDGFβ receptor inhibition of NR2B-containing NMDA receptors, which results in an inhibition of excitotoxicity caused by overstimulation of NMDA receptors. Glutamate mediated neurotoxicity in AD is

widely accepted and this hypothesis relies on the assumption that glutamate receptors such as NMDA receptors are overactivated. Since excitotoxicity is observed in AD, Aβ42 mediated inhibition of PDGF-BB activity resulting in over-activation of NMDA receptor could potentiate/worsen the excitotoxicity observed in AD. There is evidence that over-activation of extrasynaptic NMDA receptor results in Aβ production[91]. Furthermore, studies from our lab by Maryam Vasefi showed that Aβ42 inhibited PDGF-BB mediated neuroprotection against NMDA cytotoxicity. Thus, Aβ mediated inhibition of PDGF-BB activity resulting in overactivation of NMDA receptor could result in incremental Aβ production in a positive feedback manner which could result in Aβ accumulation and ongoing Aβ pathology.

The incidence of AD increases following cerebral ischemia and stroke where hypoxic conditions occur in affected brain areas. There is an increasing amount of evidence that hypoxia contributes to the pathogenesis of AD by increasing the accumulation of Aβ as well as hyperphosphorylation of tau, blood-brain barrier function impairment and promoting neuronal degeneration[92]. Hypoxia has been shown to activate intracellular death signalling pathways in neurons. To study the antiapoptotic mechanisms triggered by hypoxia, Zhang, S. X. *et al*. conducted a study where RN46A neuronal cells have been shown to induce apoptosis very late under hypoxic conditions (48hrs), indicative of neuroprotective mechanisms to protect against hypoxia induced cell death. Hypoxia induced a time-dependent increase in PDGF-B mRNA and protein expression as well as stimulated PDGFβ receptor phosphorylation. In addition, hypoxia induced a much prolonged increase in Akt phosphorylation resulting from PDGFβ receptor phosphorylation by endogenous PDGF-BB. Moreover, the induction of neuronal survival was due to endogenous PDGF-BB. In addition, the PDGF/PDGFβ receptor/Akt activation is involved

in inducing downstream hypoxia-inducible factor 1 alpha (HIF-1 alpha) gene transcription which is a survival gene induced by hypoxia. Therefore, PDGF-BB and PDGFβ receptor expression is up-regulated during hypoxia and this activation triggers downstream signalling pathways involved in neuronal survival[93]. Since Aβ42 has been shown to inhibit PDGFβ receptor activation by PDGF-BB, this inhibition can lead to lack of PDGF-BB mediated neuroprotection against hypoxia and result in increase in Aβ accumulation, tau hyperphosphorylation and blood-brain impairment resulting from hypoxia.

There is considerable evidence about the involvement of oxidative stress in AD. Oxidative stress occurs due to the net damaging effects of oxygen radicals which results in neuronal death in AD[94]. In a study by Zheng, L. *et al.*, the neuroprotective effects of PDGF against oxidative stress was evaluated using primary cultured mouse cortical neurons exposed to H_2O_2 mediated oxidative stress. PDGF-BB was shown to increase neuronal survival and suppressed H_2O_2-induced caspase-3 activation in wild type neurons, indicative of an antiapoptotic mechanism in PDGF-BB mediated neuroprotection against oxidative stress. PDGF-BB activated Akt, JNK and p-38 indicative of the involvement of these proteins in PDGF-BB mediated neuroprotection against oxidative stress. The PDGF-BB mediated neuroprotection against oxidative stress was PDGFβ receptor dependent[95]. Therefore, PDGF-BB mediated neuroprotection via PDGFβ receptor against oxidative stress includes activation of Akt, ERK, JNK and p38. In a study conducted by Cheng, B. and Mattson, M. P., exposure of rat and mouse hippocampal cell cultures to the hydroxyl radical-promoting agent $FeSO_4$ caused progressive neuronal loss. However, PDGF-BB pre-treatment attenuated $FeSO_4$-induced neuronal degeneration. $FeSO_4$ induced peroxide accumulation in neurons which was attenuated by

cultures pretreated with PDGF-BB. Moreover, PDGF-BB increased the expression of anti-oxidant enzymes catalase and glutathione peroxidase. Therefore, PDGF-BB is involved in neuroprotection against oxidative insults[37]. Aβ42 inhibition of PDGF-BB signalling involved in neuroprotection against oxidative stress could result in decrease of PDGF-BB mediated neuroprotection against oxidative stress. Thus, Aβ42 inhibition of PDGF-BB signalling could be responsible for oxidative stress observed in AD.

3.4 AB42 inhibition of downstream effectors of PDGFβ receptors

PDGF receptors mediate downstream signalling pathways which include phosphoinositode 3-kinase (PI3K)/Akt (protein kinase B (PKB))/mTOR pathway. Abnormal activation of this pathway through mutation of any of multiple genes occurs in cancer. PI3K is involved in driving cell proliferation and cell survival. Akt is found to be activated frequently in cancer cells via PI3K. Negative regulation of PI3K/Akt/mTOR pathway occurs by tumor suppressor genes. Akt is flanked by two tumor suppressors which are: 1) phosphatase and tensin homolog (PTEN) which inhibits Akt from upstream and 2) tuberous schlerosis complex 1/2 (TSC1/TSC2) heterodimer which inhibits Akt from upstream of mTOR and downstream of Akt. Phosphorylation of Akt leads to phosphorylation of TSC2 which disrupts the TSC1/TSC2 complex which leads to high levels of Rheb-GTP and activation of mTOR. mTOR activation leads to regulation of ribosome biogenesis, protein synthesis and cell growth[57]. During the course of our study, evaluation of Akt phosphorylation at site Ser473 which is a downstream effector of PDGFβ receptor revealed that Aβ42 pre-treatment of SH-SY5Y cells inhibited PDGF-BB induced Akt Ser473 phosphorylation (Figure 7). Since the PI3K/Akt pathway is involved in cell

proliferation, inhibition of Akt activation can lead to inhibition of cell proliferation leading to loss of cell growth and thus result in neurodegeneration observed in AD.

Akt phosphorylation is neuroprotective since GSK3β is inhibited by activated Akt and GSK3β mediates tau hyper-phosphorylation to prevent long-term potentiation and negatively effect learning and memory. These findings led to the "GSK hypothesis of AD" since GSK3β activation leads to the previously mentioned pathological characteristics which are characteristic of AD[96-99]. Thus, Aβ inhibition of Akt Ser473 phosphorylation through inhibition of PDGF-BB signalling could lead to activation of GSK3β which would increase tau phosphorylation, worsen memory impairment and promote neuronal apoptosis. There is increasing evidence relating the neuroprotective capability of Akt *in vivo*. In a study conducted by Jimenez, S. *et al*., the AD mouse model PS1xAPP tg was used which develops Aβ plaques during early stages (3 to 4 months) but does not undergo neurodegeneration. However, during the late stages (17 to 18 months) hippocampal neurodegeneration coupled to Aβ oligomer formation occurs. The age-dependent switch from neuroprotection to neurodegeneration was evaluated at the molecular level. GSK3β phosphorylation at Ser9 responsible for GSK3β inhibition was up-regulated in 6-month old PS1xAPP tg mice hippocampus. However, GSK3β Ser9 phosphorylation was reduced in 18-month old PS1xAPP tg mice, indicative of GSK3β activation during later stages of AD. Using N2a and primary neuron cell cultures, it was shown that the soluble amyloid precursor protein-α (sAPPα) which was the predominant APP-derived fragment in 6-month old PS1xAPP tg mice acted through the neurotrophic insulin and/or IGF-1 receptors to activate the PI3K/Akt pathway leading to GSK3β Ser9 phosphorylation for GSK3β inactivation resulting in neuroprotection. However, various oligomeric Aβ forms found in the soluble fractions of 18-

month old PS1xAPP tg mice inhibited PI3K/Akt activation leading to GSK3β activation due to lack to Akt mediated GSK3β Ser9 phosphorylation, leading to decrease in neuronal survival[100]. Thus, Aβ42 not only can inhibit Akt through PDGF-BB signalling, but also through various other neurotrophic pathways which can lead to GSK3β mediated inhibition of prosurvival pathways.

Even though various *in vivo* studies showed that Akt up-regulation may be neuroprotective in AD, there are contradictory findings with respect to Akt activity in the AD brain. In a study conducted by Griffin, R. J. *et al*. Akt activation was significantly increased in the human temporal cortex neuronal particulate fractions in AD brain samples (representing proteins in the plasma membrane of the temporal cortex). Akt is directed to the plasma membrane by PI3K resulting in subsequence Akt activation through phosphorylation. There was a total increase in activation of Akt in AD as measured by phosphorylated-Akt/total Akt ratio. In addition, particulate phospho-Akt levels were positively correlated with neurofibrillary tangle development indicative of involvement of phospho-Akt for AD pathogenesis. PTEN which is a negative regulator of Akt activation was inhibited in the AD neurons and was negatively correlated with neurofibrillary tangles and senile plaques, indicative of Akt activation being responsible for AD pathogenesis[101]. Additional studies also suggest an up-regulation of Akt activity in human AD brain where Akt activity up-regulation was correlated with neurofibrillary tangle development[102-103]. Thus, multiple studies prove a positive correlation between Akt activation and AD disease progression. Nevertheless, the relation between Akt activation and AD disease pathology is controversial. One variable to consider is that AD mouse models showed neuroprotective potential for Akt activation whereas human AD brain models showed

neurodegenerative potential for Akt activation. Ideally, human AD brain models could more accurately portray what happens in a human AD brain. However, to avoid controversy perhaps more studies should be conducted with the same models which should ideally be human AD brain models to evaluate the relation between Akt activation and AD disease pathology.

3.5 Does Aβ42 inhibition of PDGF-BB signalling lead to neurotoxicity

Previously, studies in the Beazely lab showed that PDGF-BB failed to be neuroprotective against Aβ42 for SH-SY5Y cells and primary neurons. In our studies, 1ng/ml PDGF-BB treatment of SH-SY5Y cells for 5 min increased PDGFβ receptor Tyr1021 phosphorylation by 155 fold and co-treatment with 1ng/ml PDGF for 5 min and 5μM Aβ42 for 10 min increased Tyr1021 phosphorylation by 94 fold. Even though there is a significant inhibition of PDGF-BB mediated PDGFβ receptor activation by Aβ42, there is still significant increase in receptor activation when SH-SY5Y cells are co-treated with Aβ42 and PDGF-BB. However, even though there is still a significant increase of PDGF receptor activation by PDGF-BB in presence of Aβ42, PDGF-BB mediated PDGFβ receptor activation still fails to be neuroprotective against Aβ42 toxicity. This may suggest that Aβ42 was lethal to neurons in a mechanism distinct from PDGF-BB signalling. Therefore, although Aβ42 inhibits neurotrophic PDGF-BB mediated PDGFβ receptor activation, this is not the primary mechanism through which Aβ42 was lethal to SH-SY5Y cells and primary neurons. In addition, PDGFβ receptor activation is not sufficient to protect against Aβ42 toxicity. Nevertheless, in our studies, Aβ42 mediated inhibition of PDGF-BB signalling also led to inhibition of Akt activation which is involved in cell proliferation and cell survival. Furthermore, previous studies from our lab revealed Aβ42 inhibited PDGF-BB

protection against NMDA cytototoxicity on SH-SY5Y cells, indicative of Aβ42 mediated inhibition of PDGF-BB signalling to prevent PDGF-BB protection against NMDA toxicity (Figure 11). Therefore, Aβ42 mediated inhibition of PDGF-BB signalling contribute partially although not significantly to the cell death caused by Aβ42 toxicity.

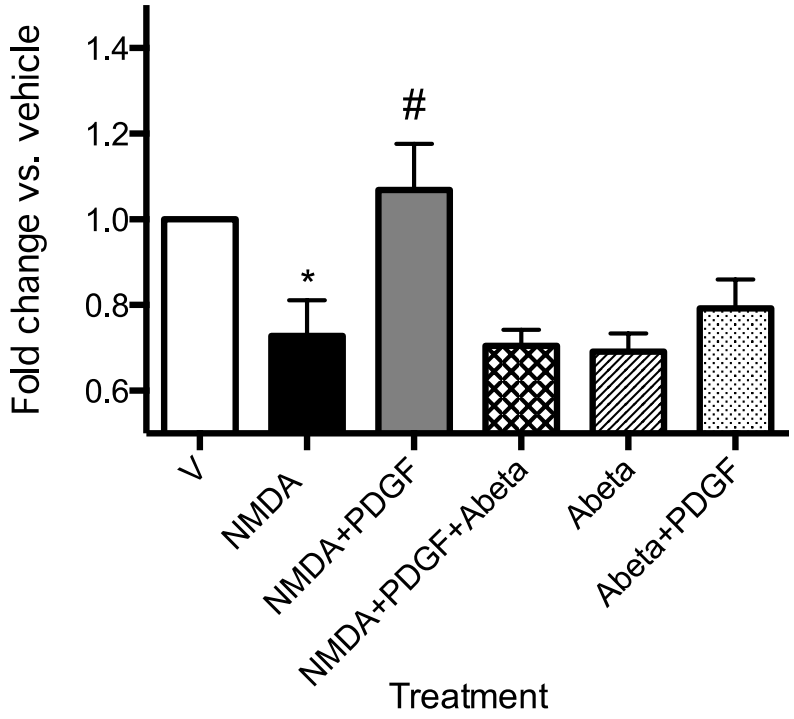


Figure 11: β-amyloid prevents PDGF-BB-induced neuroprotection against NMDA excitotoxicity. SH-SY5Y cells were treated with 100 μM NMDA/1 μM glycine, 10 ng/mL PDGF-BB, or 5 μM β-amyloid alone or in combination for 10 min. Each condition was applied in triplicate. Cells were then incubated for 24 h before the remaining cell number was measured using the MTT assay. Cell viability was expressed as fold absorbance (change) relative to the cells treated with vehicle alone. Data represent the mean and standard error of 7-8 independent experiments. * $p < 0.05$ compared to vehicle, # $p < 0.05$ compared to NMDA alone, one way ANOVA with Tukey's multiple comparison test.

3.6 Future directions.

In order to validate for the presence of interaction between Aβ42 and PDGF-BB, perhaps a different well renowned antibody such as 6E10 antibody should be used to immunoprecipitate Aβ42, after which western blot can be used to evaluate whether PDGF-BB interacted with the immunoprecipitated Aβ42. Furthermore, if Aβ42 does not interact with PDGF-BB to inhibit PDGF-BB signalling, it would be worthwhile to evaluate whether Aβ42 inhibits PDGFβ receptor activation by blocking serotonin (5-HT) mediated transactivation of PDGFβ receptor[104]. Since Aβ42 inhibited Akt activation through BDNF and NGF pathway which activate TrkB and TrkA receptors respectively from a different study, it would be worthwhile to identify whether Aβ42 inhibits TrkA and TrkB phosphorylation to prevent BDNF and NGF signalling which are both neurotrophic factors. In addition, in that study, Aβ42 also inhibited insulin and IGF-1 signalling which are both neurotrophic factors and ligands for RTKs, indicative of Aβ42 mediated inhibition of neurotrophic signalling[100]. Therefore, it would be worthwhile to identify whether Aβ42 inhibits other RTK neurotrophic signalling pathways, such as the neurotrophin-3 (NT-3) pathway which is a ligand for the RTK TrkC and neurotrophin-4/5 (NT-4/5) pathway which is a ligand for the RTK TrkB. Therefore, a novel hypothesis can be proven where Aβ42 inhibits multiple neurotrophic signalling pathways to inhibit cell survival and cell growth leading to cell death. Furthermore, the mechanism of Aβ42 inhibition of neurotrophic signalling could be elucidated by evaluating for the presence of interactions between Aβ42 with the RTK and/or the neurotrophic factors since such interactions could prevent interaction between the ligand and the receptor leading to loss of ligand activity. In addition, if there is an interaction between Aβ42 and the RTK/ligand and there is a modulation of receptor activation by Aβ42, the effect of such

modulation of receptor activation on downstream effectors AKT, extracellular signal-related kinase (ERK) and glycogen synthase kinase 3 (GSK3) should be evaluated.

Chapter 4: Materials and methods

4.1 SHSY-5Y cell culturing

The SHSY-5Y human neuroblastoma cell line which is a present from University of Nebraska by Dr. Shilpa Buch and colleagues was supplemented with DMEM/F-12 media (1:1) containing glutamine and HEPES (Fisher). 10% fetal bovine serum (Sigma) was added to the media and the cell line was maintained in 5% CO_2 at 37^0C. The cells were serum starved by replacing the media with 10% fetal bovine serum with just media for 24hrs before treating the cells with various drugs.

4.2 Aβ42 oligomer preparation:

Stine`s method with a slight modification was used for preparation of Aβ42 oligomers[105]. Aβ42 (rPeptide, Georgia, USA) was dissolved to 1mg/ml by immersing the peptide in 100% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) and aliquoted. HFIP was vacuumed out and the peptide without the HFIP was stored at -20^0C. Before usage of Aβ42, the Aβ42 was dissolved by DMSO to 5mM, sonicated for 10 minutes, diluted to 100µM with serum free media and incubated at 4^0C for 24 hrs prior to treatment.

4.3 Western Blot:

Cells were exposed to drug, after which they were washed with cold PBS. The cells were lysed with cold lysis buffer (150mM NaCl, 1mM EGTA, 1mM EDTA, 30mM sodium

pyrophosphate, 1mM β-glycerophosphate, 20mM Tris-HCl pH 7.5, 1mM sodium orthovanadate and 1% triton-X; Halt protease and phosphatase inhibitor (Fisher) was added to the lysis solution before use). The cells were removed from the wells with a scraper, homogenized and centrifuged at 14,000g for 20 minutes at 4^0C. The supernatant with the proteins were collected and the cells were discarded. The proteins were separated through SDS PAGE and the proteins were transferred from the polyacrylamide gel to the nitrocellulose membrane, blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 1hr at room temperature or overnight at 4^0C. The membranes were incubated with the primary antibody for 1 hr at room temperature or overnight at 4^0C, followed by washing the membranes 3 times with TBST with 10 min intervals between each wash. The membranes were incubated with secondary antibody conjugated with horseradish peroxidase for 1 hr at room temperature, washed 3 times with TBST with 10 min intervals, and finally exposed with a chemiluminescent substrate (Millipore) to visualize positive interaction between primary antibody and target protein. The visualization and imaging were done using Kodak 4000MM Pro Imaging Station and the Kodak Molecular Imaging Software were used for densitometric analysis. Membranes were stripped and used again for screening with other antibodies.

4. 4 Immunoprecipitation:

For immunoprecipitation, 3 wells per 6 well plate were used for one sample. The cells in the wells were drug treated, washed in ice cold PBS, and then lysis buffer (ingredients same as lysis buffer used for western blot except contained NP-40 1% instead of 1% Triton-X) were added and the rest of cell lysis protocol followed that of the same one used for western blot. A

BCA protein assay was performed to measure total protein concentration in the cell lysate. The total protein should be 1-2µg/µl. The samples were then normalized to the same protein concentration by adding additional lysis buffer. Equal volume of cell lysate from each sample was transferred to a fresh microfuge tube. The volume contained 100-1000µg of total protein. 30µg from each sample were transferred to fresh microfuge tubes as lysate control, after which loading buffer were added to the samples and stored at -20^{0}C.

Next, 1.0µg of control IgG protein together were added with 40µl of suspended 25%v/v protein-A/G agarose conjugate for each sample and incubated at 4^{0}C for 1-2hrs. The antibody-agarose conjugate were centrifuged at 1,000g for 5 minutes at 4^{0}C. The precleared pellet was saved. 30µl of 3X loading buffer were added to the pellet and stored at -20^{0}C.

The supernatant or approximately 100-1000µg of total cellular protein for each sample were transferred to fresh microfuge tubes and 0.2-2µg of primary antibody were added to each sample and incubated overnight at 4^{0}C shaking. 40-50µl of appropriate antibody-agarose conjugate were added to the protein samples and incubated at 4^{0}C on a rocker or rotating device for 1hr to overnight. The samples were centrifuged at 1,500g for 5minutes at 4^{0}C and the supernatants were transferred to new microfuge tubes. 40µl of the supernatant were added to 20µl 3X loading buffer and stored at -20^{0}C. The pellets were washed 2-4 times with 1ml PBS and after each wash; the centrifugation step would be repeated. After the final wash, the supernatant was discarded and the pellet was resuspended in 30µl of 3X loading buffer. The samples that are in loading buffer were stored at -20^{0}C until they are used.

The samples were boiled at 100^{0}C for 5 minutes and electrophoresed and immunoblotted. For each sample, there were four different variations of the sample running: 1) lysate control

mentioned in 1st paragraph, 2) a precleared pellet mentioned in 2nd paragraph, 3) supernatant and 4) pellet mentioned in the 3rd paragraph.

This protocol was followed for immunoprecipitation experiments involving PDGFβ receptor isolation for evaluating PDGFβ receptor and Aβ42 interaction since SH-SY5Y cells expressed sufficient protein levels of PDGFβ receptor to be used for immunoprecipitations. However, SH-SY5Y cells did not express sufficient protein levels of PDGF-BB and thus SH-SY5Y cells were not used for immunoprecipitation. However, this protocol was modified such that PDGF-BB diluted from stock solution to 1ng/ml and 5µg/ml and Aβ42 diluted from stock to 5µM and 1µM were used to evaluate interactions between PDGF-BB and Aβ42. For this modified immunoprecipitation, the new protocol followed all the steps starting from the 3rd paragraph of the original immunoprecipitation protocol discussed above. Therefore, for this modified immunoprecipitation, there were no lysate of precleared pellet samples. There were only supernatant and pellet of samples.

Chapter 5: Conclusion

Aβ42 inhibits PDGF-BB mediated PDGFβ receptor activation at multiple sites including Tyr1021, Tyr740, Tyr751 and Tyr771. In addition, Aβ42 inhibited PDGF-BB mediate PDGFβ receptor Tyr1021 phosphorylation in a dose-dependent manner. PDGF-BB is neuroprotective against multiple insults including ischemia, oxidative stress, glucose deprivation and glutamate/NMDA receptor excitoxicity. Nevertheless, PDGF-BB failed to protect against Aβ42 toxicity. Aβ42 mediated inhibition of PDGF-BB signalling led to inhibition of Akt activation which is involved in cell proliferation and cell survival. Aβ42 mediated inhibition of PDGF-BB signalling was not through interaction of Aβ42 with PDGFβ receptor, but perhaps through interactions between Aβ42 with PDGF-BB ligand which a conclusion inconclusive at this point. Although Aβ42 inhibits PDGF-BB signalling, there is still significant PDGFβ receptor activation. Nevertheless, the significant PDGFβ receptor activation is not sufficient for PDGF-BB to be neuroprotective against Aβ42 toxicity. Aβ42 mediated inhibition of PDGF-BB signalling could contribute to Aβ42 toxicity.

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