Angiogenin as a Multi-Targeted Therapy for Alzheimer's Disease

Sang Hyun Kim, Donna Leonardi

Abstract— Angiogenin, a potent agonist of neovascularization, has been implicated in the pathogenesis of various neurodegenerative disorders, including Parkinson's disease (PD) and amyotrophic lateral sclerosis (ALS). A recent study showed that the serum level of angiogenin is directly correlated with the cognitive functions of patients with Alzheimer's disease (AD), suggesting that angiogenin may play a role in AD as well. However, its mechanism of action independent of its regulatory effects on neovascularization is unknown. This study investigated angiogenin's possible mechanism of action in an *in vitro* model system of AD and proposes the potential of angiogenin to be used as a multi-targeted therapy that inhibits the level of phosphorylated tau and beta-amyloid (Aß) production, both of which play critical roles in the progression of the disease. Results show that angiogenin reduced tau phosphorylation and Aß production with statistical significance ($p<0.05$) through a glycogen synthase kinase 3ß (GSK3ß)-dependent pathway. Angiogenin may therefore be a viable multi-targeted therapeutics strategy, especially with the current lack of treatment options available for AD.

Index Terms— AKT, Alzheimer's disease, Angiogenin, Beta-amyloid, Glycogen synthase kinase 3ß, Multi-targeted therapy, Tau

1 INTRODUCTION

Alzheimer's disease (AD) is the most common neurodegenerative disorder that is characterized by a progressive cognitive decline. It is clinically identified by the presence of beta amyloid plaques and neurofibrillary tangles (NFT), which cause neuronal dysfunction and cell death. The pathological basis of the disease still remains unclear, resulting in a lack of viable treatment options. Present medications are palliative measures at best, that only delay the aggravation of symptoms.

Neurofibrillary tangles and beta amyloid plaques are formed by aggregations of tau and beta amyloid (Aß) proteins, respectively. NFT and Aβ plaques both have critical roles in the progression of the disease, though the inhibition of only one of the proteins has been found to be ineffective. Aß-targeted monotherapies have shown potential in *in vitro* testing, but have not demonstrated the desired effects *in vivo*, failing in Phase III clinical trials [2]. Tau-targeted therapies have also not shown enough promise to be continued in clinical trials [5]. Thus, it seems advantageous to seek a multi-targeted therapy that can suppress levels of both Aß production and phosphorylated tau.

Angiogenin, a liver-derived angiogenic factor, has implications with various neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) [10, 12]. Wild-type angiogenin reduced neuronal death and delayed motor dysfunction in mouse models of ALS [10]. Similarly, *in vitro* models of PD showed a neuroprotective role of exogenous angiogenin against neurotoxin MPP+ and rotenone, which induce Parkinsonian syndrome in animals and humans [12]. *In vitro* findings such as this are significant as they suggest roles of angiogenin independent of neovascularization. The neuroprotective effects of angiogenin in both ALS and PD are suggested to be mediated by the phosphorylation of AKT. In an ALS model system, inhibition of AKT prevented angiogenin's neuroprotective effect [10]. While the exact mechanism of action is not fully elucidated, it is suggested that angiogenin induced AKT signaling may inhibit downstream targets, such as caspase-9 and FOXO, subsequently inhibiting apoptosis [12].

Angiogenin is also being considered as a potential biomarker of AD and a predictor of the progression of the disease. A recent study reported that serum angiogenin levels were significantly lower in AD patients and that angiogenin levels were correlated with the patients' cognitive functions [11]. However, there is scant literature investigating the role of angiogenin in AD, unfortunate, as it may play a critical role in a biological pathway that regulates cognition.

While angiogenin's role in the activation of AKT needs further elucidation, the significance of pAKT in AD is already known. Phosphorylated AKT inhibits glycogen synthase kinase 3ß (GSK3ß) specifically through the phosphorylation of Ser9, which has been previously suggested to directly regulate both Aß production and tau phosphorylation [6, 8]. Thus, it seems of significance to investigate the role of angiogenin in the inhibition of GSK3ß with significant impact on tau phosphorylation and Aß production. If angiogenin plays a significant role in the reduction of ptau and Aß production, it may have a potential to be used as a multi-targeted therapy for AD.

In order to investigate this, N2a-695 (a mouse neuroblastoma cell line transfected to express human Aß) was treated with exogenous angiogenin. It is hypothesized that exogenous angiogenin would activate AKT, increasing the phosphorylation and deactivation of GSK3ß, and subsequently reduce the production of Aß and tau phosphorylation. Furthermore, angiogenin was administered in combination with a GSK3ß inhibitor to N2a-695 in order to confirm the proposed GSK3ß–dependent mechanism.

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2 PROCEDURE FOR PAPER SUBMISSION

2.1 Cell Culture

N2a-695, a stably transfected mouse neuroblastoma cell line to express human Aß and ptau, was obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA). Cells were cultured in 50/50 Reduced Serum Medium (Invitrogen, Carlsbad, California, USA) and Dulbecco's Modified Eagle's Medium (Invitrogen), supplemented with 5% fetal bovine serum (Invitrogen) and 1% penicillin-streptomycin (Invitrogen). Cells were incubated at 37°C and 5% CO_2.

2.2 Exogenous Angiogenin Treatment

N2a-695 cells were treated with various concentrations of exogenous angiogenin (0.39-12.5 nM) diluted in 0.2 µm- filtered phosphate buffered saline (Invitrogen) containing 0.1% bovine serum albumin (Invitrogen). Concentrations were established after two-fold serial dilutions.

2.3 Assays

MTS Colorimetric Cell Proliferation assay

N2a-695 cells were seeded at a density of 15,000 cells per well of a 96-well assay plate and incubated for 24 hours. After treatment, 15 µL of CellTiter 96® AQueous One Reagent (Promega, Madison, Wisconsin, USA) was added to each well. The well plate was subsequently incubated for 1 hour at 37°C in a humidified, 5% CO2 condition. Absorbance was read using a microplate reader (BioTek, ELx808, Winnoski, Vermont, USA) at 490 nm.

Preparing lysates

After treatment, cells were trypsinized and transferred to 15mL centrifuge tubes (Corning Inc., Corning, New York, USA). The cells were spun at 1000 rpm for 7 minutes to form a pellet. For every $1x10^6$ cells, 1.5mL of ice-cold 1x PBS (Invitrogen) was used to rinse the cells. After the cells were re-centrifuged at 1000 rpm for 7 minutes, PBS was decanted. 1mL of lysis buffer (Promega, Madison, Wisconsin, USA) and 10 µL of protease inhibitor cocktail (Sigma) was added for every $1x10^6$ cells and immediately moved to microcentrifuge tubes. They were kept on ice for 10 minutes and then centrifuged at 13,000 rpm for 15 minutes. The supernatant was transferred to new prechilled microcentrifuge tubes, which were then stored at -80°C.

PathScan® Phospho-AKT ELISA (Cell Signaling Technology, Danvers, Massachusetts, USA)

A 96-well assay plate was coated as per company's protocols. Briefly, the Phospho-AKT ELISA was conducted by adding 100 µL of prepared lysates to each well and incubating the plate for 2 hours at 37°C. After the plate was aspirated and washed, the detection antibody was diluted as per protocol and added (100µL) to each well. The plate was sealed and incubated at 37°C for 30 minutes and washed. 100µL of TMB Substrate was added to each well at 37°C for 10 minutes and the plate was read using a microplate reader at 450 nm after the stop solution was added.

GSK3ß (Phospho-Ser9), Phospho-Tau, beta-amyloid assay (KPL, Gaithersburg, Maryland)

Cell lysates (100µL) were added to wells of a 96-well assay plate, which was then incubated at 4°C for 24 hours. The plate was aspirated and blocked by adding 300 µL of the blocking solution to each well. After aspirating the plate, the respective antibody (Abcam, Cambridge, MA) was diluted and added (100µL) to each well. The plate was incubated for 1 hour at room temperature and washed. 100 µL of diluted secondary antibody was subsequently added to each well. After the plate was incubated for an additional 1 hour at room temperature, 100µL of substrate solution was added to each well. The absorbance was read at 405 nm.

Caspase 3/7 Assay (Promega)

75 µL of Caspase-Glo Reagent, prepared as per protocol, was added to each well seeded with 15,000 cells per well. The plate was incubated for 1 hour at 37°C and 5% CO_2 and the luminescence of the samples was measured (Synergy HT; Winnoski, Vermont, USA). Results were corrected with cell viability in order to account for the variance in number of cells among the samples treated with different concentrations of angiogenin.

GSK3ß Inhibitor pretreated Aß, phospho-tau, cell viability, caspase-3 assays

There are several pathways that regulate Aß production, level of tau phosphorylation, cell proliferation, and caspase-3. To determine whether the observed changes were due to the proposed GSK3ß-dependent pathway, the assays were conducted with lysates prepared from cells treated with varying concentrations of angiogenin (0.39-12.5 nM) after 1-hour pretreatment with GSK3ß inhibitor (Sigma, St. Louis, Missouri, USA), SB216763 (10µM).

2.4 Data Analysis

Data was analyzed using Excel (Microsoft, Redmond, Washington, USA). The recorded data was corrected to percent of control and t-test was computed to determine statistical significance of data with alpha=0.05. Assays were conducted with n≥3 and repeated at least three times.

3 RESULTS

Effect of angiogenin on the phosphorylation of AKT.

pAKT plays a crucial role in the pathogegnesis of AD by inhibiting the activity of GSK3ß, an enzyme that positively regulates both Aß production and tau phosphorylation. It has been reported that angiogenin phosphorylates AKT in ALS and PD *in vitro* models [10, 12], but the effect of angiogenin on pAKT has not been investigated in AD. Therefore, it was of interest to measure the changes in the level of pAKT after N2a-695 cells were treated for 24-hour with angiogenin. The range of angiogenin concentrations was determined after MTS cell viability assays were conducted. The results showed that the level of pAKT increased after angiogenin (0.78-6.25nM) treatment

with statistical significance ($p<0.05$) when compared to control.

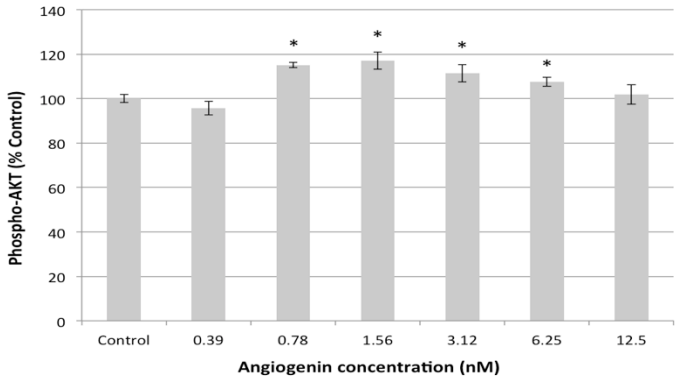

Fig 1. The effect of angiogenin on pAKT. The level of pAKT increased after angiogenin treatment of concentrations between 0.78nM and 6.25nM. Bars are means ±SD (n=3). *($p<0.05$) compared with the solvent control.

Effect of angiogenin on phosphorylation of GSK3ß.

The finding that angiogenin upregulates AKT phosphorylation in AD suggested that the reported correlation between angiogenin and cognitive functions may be due to its possible regulatory effect on Aß production and ptau via inhibition of GSK3ß activity. To determine whether angiogenin may cause a significant change in the level of phosphorylated and inactivated GSK3ß an ELISA was conducted to measure pGSK3ß after 24-hour of angiogenin treatment. Angiogenin, at concentrations between 0.78 and 6.25 nM increased the level of pGSK3ß with statistical significance ($p<0.05$).

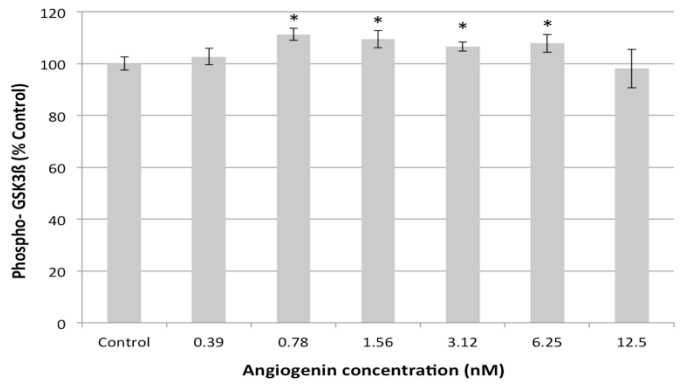

Fig 2. The effect of angiogenin on phospho-GSK3ß. Angiogenin (0.78nM-6.25nM) increased the level of phosphorylated GSK3ß. Bars are means ±SD (n=3). *($p<0.05$) compared with the solvent control.

Effect of angiogenin on Aß production and tau phosphorylation.

ELISA was conducted to measure Aß production and tau phosphorylation after angiogenin treatment. As predicted, based on the finding that angiogenin increases the level of pGSK3ß, Aß production decreased with statistical significance ($p<0.05$) at angiogenin concentrations between 0.39 and 12.5nM. Maximum inhibition of Aß production was at concentrations between 0.78 and 6.25 nM, which correspond to points at which increases in pAKT and pGSK3ß were significant. Similarly, angiogenin decreased the levels of ptau with statistical significance ($p<0.05$) at concentrations between 0.78 and 12.5 nM. This result highlights the potential of angiogenin to be used as a multi-targeted therapeutic option that can suppress both Aß production and tau phosphorylation.

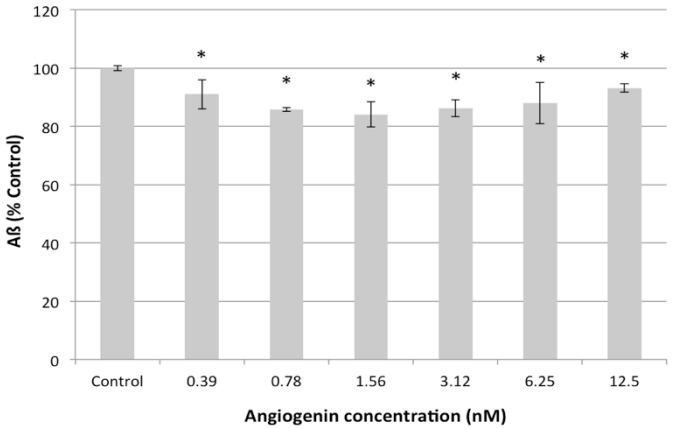

Fig 3. The effect of angiogenin on Aß production. Level of Aß production decreased after angiogenin (0.39nM-12.5nM) treatment. Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

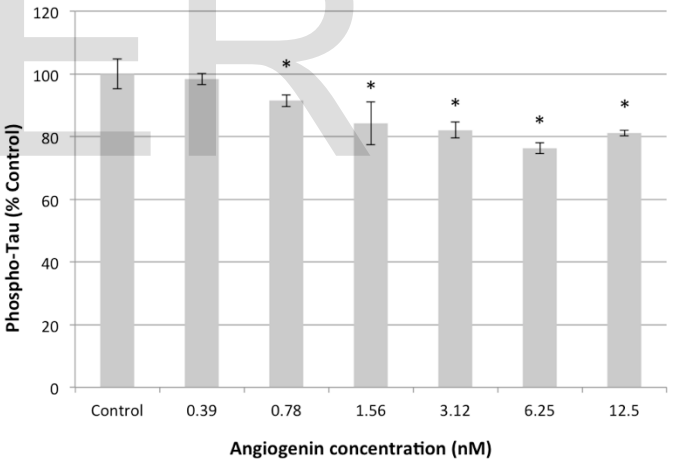

Fig 4. The effect of angiogenin on ptau. Tau phosphorylation decreased after angiogenin (0.78nM-12.5nM) treatment. Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

Effect of angiogenin on cell viability and caspase 3/7.

Other than neuronal dysfunction, Aß production and ptau lead to cell death by induction of apoptosis. To determine whether the inhibitory effects of angiogenin on the phosphorylation of tau and Aß production coincides with a reduction in neuronal death, assays were conducted to measure the effect of angiogenin on N2a-695 cell viability and caspase 3/7, an apoptotic enzyme. At concentrations between 0.78nM and 6.25 nM, angiogenin increased cell viability with statistical significance ($p<0.05$) when compared to control. Caspase 3/7 decreased with statistical significance ($p<0.05$) at concentra-

tions between 0.39nM and 12.5nM of angiogenin.

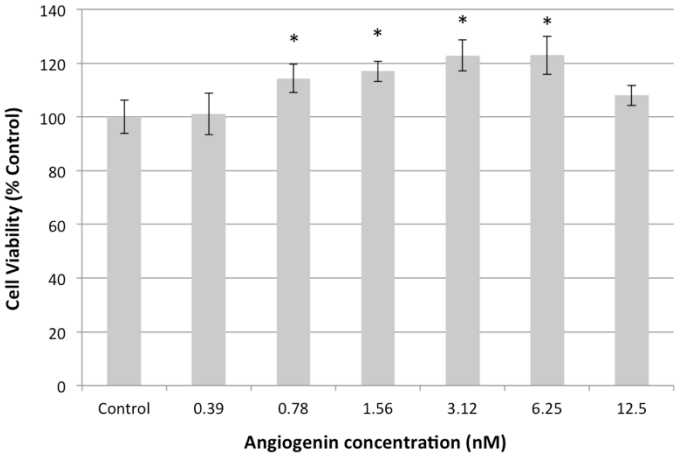

Fig 5. The effect of angiogenin on N2a-695 cell viability. Cell viability increased after angiogenin treatment at concentrations between 0.78nM and 6.25nM. Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

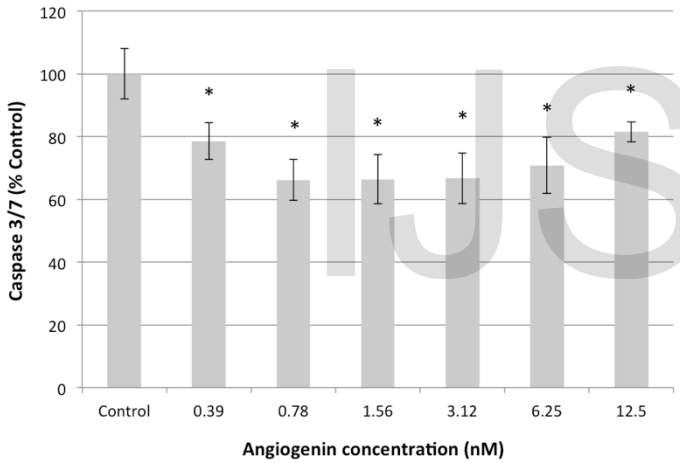

Fig 6. The effect of angiogenin on caspase 3/7. Angiogenin (0.39nM-12.5nM) decreased caspase 3/7 Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

Effect of angiogenin on SB216763 pretreated N2a-695

There are other pathways that regulate the levels of Aß production, ptau, cell proliferation, and caspase 3/7. In order to confirm that the effects of angiogenin are due to its inhibition of GSK3ß activity, angiogenin was administered to N2a-695 cells pretreated with GSK3ß inhibitor, SB216763 (at concentration 10µM), for 1 hour. N2a-695 cells treated with 10µM of SB216763 had 80% yield of Aß production and 91% yield of tau phosphorylation when compared to the solvent control (graphs not shown). Results demonstrate no statistically significant findings with regard to the phosphorylation of tau, Aß production, caspase production or cell viability ($p<0.05$), suggesting that angiogenin's effects are dependent on the inhibition of GSK3ß, as there are no further modulations through any alternative pathways when angiogenin is added to the SB216763-treated cells.

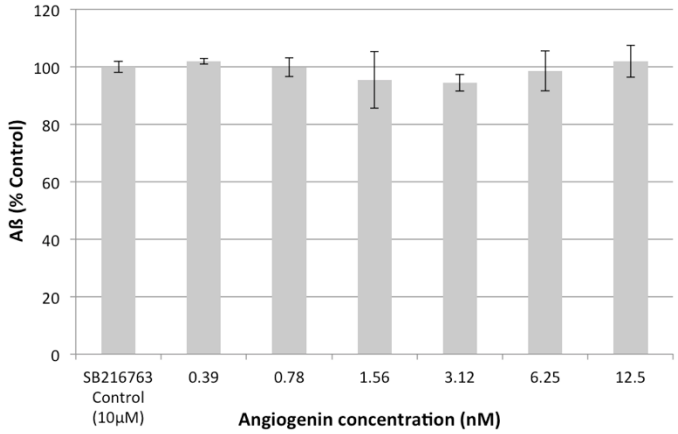

Fig 7. The effect of angiogenin on Aß production in SB216763-pretreated N2a-695. Changes to Aß production after angiogenin treatment were not statistically significant. Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

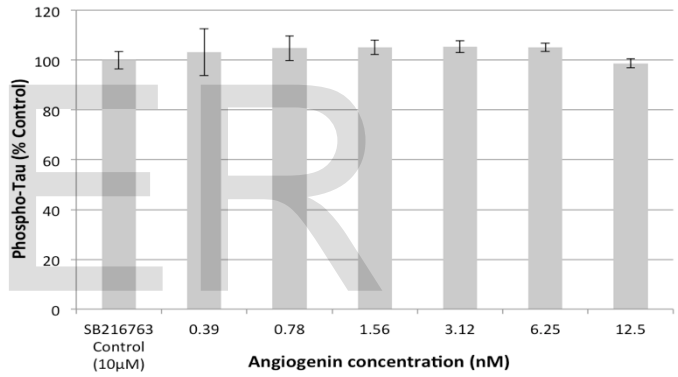

Fig 8. The effect of angiogenin on ptau in SB216763-pretreated N2a-695. Changes to ptau after angiogenin treatment were not statistically significant. Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

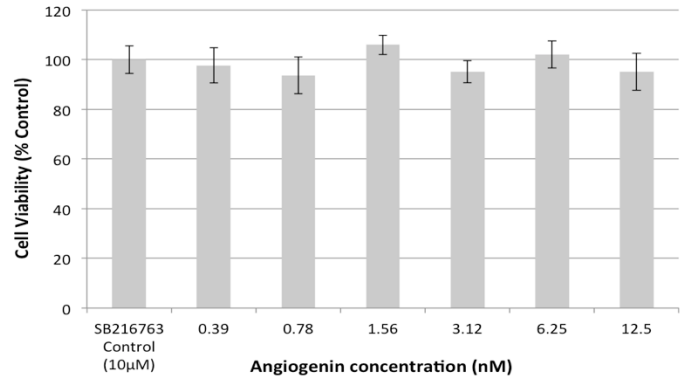

Fig 9. The effect of angiogenin on SB216763-pretreated N2a-695 cell viability. Changes to cell viability after angiogenin treatment were not statistically significant. Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

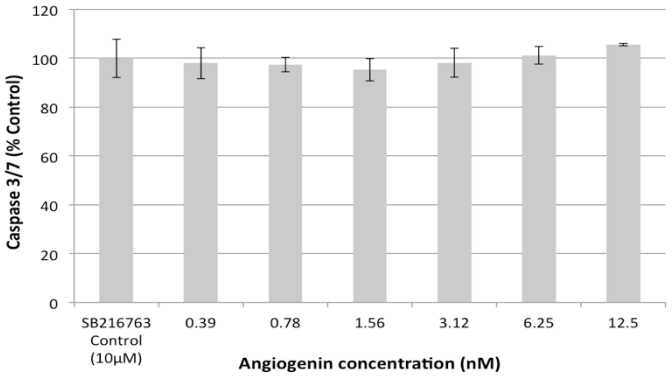

Fig 10. The effect of angiogenin on caspase 3/7 activity in SB216763-pretreated N2a-695. Changes to caspase 3/7 after angiogenin treatment were not statistically significant. Bars are means ±SD (n=5). *p<(0.05) compared with the solvent control.

4 Discussion

In this study, the mechanism of action of angiogenin in an Alzheimer's disease model was investigated. Angiogenin has received great attention for its neuroprotective effects in other neurodegenerative disorders, including Parkinson's disease and amyotrophic lateral sclerosis [10, 12]. It has also been considered as a novel biomarker of AD, as a study reported that the serum levels of angiogenin are directly correlated to AD patients' cognitive functions [11]. Understanding the mechanism of angiogenin in an AD model, may reveal pathways that can be therapeutically regulated to improve cognition. Based on studies that have suggested that the neuroprotective effects of angiogenin in PD and ALS are mediated by the activation of AKT [10, 12], it was hypothesized that angiogenin would increase the phosphorylation of AKT in N2a-695. AKT plays a critical role in AD pathogenesis by regulating GSK3ß, a novel crosstalk between Aß and tau signaling [6, 8]. Therefore, the effects of angiogenin on GSK3ß phosphorylation, Aß production, tau phosphorylation, and their associated neurodegenerative effects were measured.

Results show that the level of phosphorylated AKT increased after angiogenin treatment at concentrations between 0.78nM and 6.25nM, suggesting that angiogenin does activate AKT in AD model systems (Fig 1). As hypothesized, an increase in phosphorylated AKT corresponded with an increase in the level of phosphorylated, or inactive, GSK3ß (Fig 2). Angiogenin decreased the level of tau phosphorylation at concentrations above 0.39nM, and also reduced Aß production at all concentrations (Fig 3, 4).

Aß and tau phosphorylation have various neurodegenerative effects, specifically the induction of apoptosis in neurons. In order to confirm that the inhibition of tau phosphorylation and Aß production resulted in a reduction of this neurodegenerative effect in the AD model, N2a-695 viability and caspase 3/7 were measured. Results show that angiogenin (0.78-6.25 nM) increases cell viability and decreases caspase-3 (Fig 5, 6). This suggests that angiogenin reduces the induction of apoptosis and promotes cell proliferation.

There are other pathways, however, through which Aß production and tau phosphorylation can be regulated. In order to determine whether the effects of angiogenin in N2a-695 were due to the proposed GSK3ß-dependent pathway, the cells were pretreated with a selective GSK3ß inhibitor, SB216763, for 1 hour before angiogenin was administered. If the decrease in Aß production and phospho-tau were due to the inhibition of GSK3ß activity, angiogenin treatment on SB216763 pre-treated cells should not show any significant changes in levels of Aß and phospho-tau. Consequently, cell viability and level of caspase 3/7 should have no statistically significant changes as well. The results show that there were no significant changes in all assays, highly suggesting that the mechanism of action of angiogenin is dependent on the inactivation through the phosphorylation of GSK3ß (Fig 7, 8, 9, 10).

Since GSK3ß regulates both Aß and phosphorylated tau levels, selective GSK3ß inhibitors have been of great interest to researchers. As hypothesized, GSK3ß inhibitors decreased Aß production and tau phosphorylation in *in vitro* AD models. However, they produced adverse side effects in animals [7], damaging their potential to be used as future therapeutic options for AD patients. Despite its inhibitory effect on GSK3ß activity shown in this study, angiogenin has shown safety and tolerability in clinical trials of ALS [13]. This is subject to future research, but it is implied that angiogenin may be a novel GSK3ß inhibitor without adverse side effects.

This study analyzed the possible mechanism of action of angiogenin in a model system of AD. By regulating AKT phosphorylation, angiogenin inhibits GSK3ß activity, and thus decreases Aß production and tau phosphorylation. Angiogenin's ability to reduce neurodegenerative effects of Aß and phosphorylated tau is clearly highlighted by its induction of cell proliferation and decrease in caspase 3/7. Based on this mechanism of action, angiogenin shows clear potential to be used as a multi-targeted therapy for Alzheimer's disease. Furthermore, the following proposed pathway through which angiogenin regulates neurodegeneration can also aid in the development of other therapeutics agents of AD in the future.

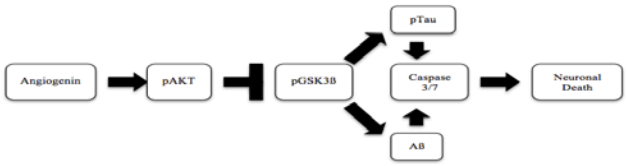

Fig 11. Schematic of angiogenin's possible mechanism of action in AD

5 Conclusion

Previously, the mechanism of angiogenin in Alzheimer's disease remained unclear. While it was reported that angiogenin could regulate cognitive function [11], the pathway involved was unknown. This study presents a possible GSK3ß-

dependent mechanism of action of angiogenin in Alzheimer's disease. Based on this role, it was concluded that angiogenin may have a potential to be used as a widely sought after multi-targeted treatment that can suppress levels of both Aß production and tau phosphorylation. This is validated by angiogenin's inhibitory effect on Aß production and ptau. It was also confirmed that their suppressions lead to a reduction in cellular apoptosis. Furthermore, the safety that angiogenin has demonstrated in ALS clinical trials [13], further supports the idea that angiogenin can be used as an AD treatment.

However, because this paper is the first to investigate the angiogenin's mechanism of action independent of neovascularization in AD, further testing using both different *in vitro* models and *in vivo* models is necessary in order to confirm these results. While studies of ALS have shown that angiogenin can be administered by intraperitoneal injections [10], it will be more ideal to regulate the endogenous concentration of angiogenin. Thus, future experimentation will analyze the use of different gene therapies and angiogenin agonists that can upregulate the level of endogenous angiogenin. For example, Vitamin D has recently been suggested to modulate endogenous angiogenin level in AD [9]. Thus, cost-effective alternatives such as regular consumption of Vitamin D may be studied in the future.

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