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Role of Amyloid Precursor Protein in Neuroregeneration on an In Vitro Model in Alzheimer's Patient-Specific Cell Lines

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ROLE OF AMYLOID PRECURSOR PROTEIN IN NEUROREGENERATION
ON AN *IN VITRO* MODEL IN ALZHEIMER'S PATIENT-SPECIFIC CELL
LINES

by

LINA SOFIA BEDOYA MARTINEZ

A thesis submitted in partial fulfilment of the requirements for the Honors in the Major Program
in Biomedical Sciences in the College of Medicine and in the Burnett Honors College at the
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ABSTRACT

Alzheimer's disease (AD) leads to neurodegeneration resulting in cognitive and physical impairments. AD is denoted by accumulation of intracellular neurofibrillary tangles, known as tau, and extracellular plaques of the amyloid beta protein ($A\beta$). $A\beta$ results from the proteolytic cleavage of the amyloid precursor protein (APP) by [Symbol]- and [Symbol]-secretases in the amyloidogenic pathway. Although, $A\beta$ has been widely studied for neurodegeneration, the role of APP in both, the healthy and diseased conditions, has not yet been entirely understood. The function that APP has in neural stem cell (NSC) proliferation, differentiation, and migration during adult neurogenesis has been previously studied. Additionally, APP has been shown to be overexpressed after neural damage resulted from conditions, such as AD and traumatic brain injury (TBI). In this study, the role of APP in *in vitro* damaged neural tissue cells was further investigated by evaluating neural progenitor cell proliferation, migration, and differentiation after a scratch assay. For these purposes, induced pluripotent stem (iPS) cells from AD patients were differentiated into neural progenitor cells to model the disease conditions and later treated with Phenserine to reduce their levels of APP expression. The results suggested that APP may enhance neural progenitor cell proliferation and glial differentiation while inhibiting neural progenitor cell migration and neuronal cell specialization after neural tissue damage.

DEDICATION

I want to dedicate this thesis to my dear aunt, Olga, whose constant care and guidance have allowed me to continue finding fruitful results along my path.

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INTRODUCTION

Approximately 5.7 million Americans aged ≥ 65 were diagnosed with Alzheimer's Disease (AD) on 2018, making it the 5th most common cause of death in the United States [1]. AD is a neurodegenerative disease that causes progressive cognitive impairment in earlier stages and gradual physiological dysfunction in later stages. There are not yet specific treatments to prevent or cure AD. However, current research efforts focus on further understanding the pathological characteristics of AD, and how these can be targeted for future treatments. Besides neurodegeneration, AD is also characterized by accumulation of intracellular neurofibrillary tangles and extracellular senile plaques [1,2]. The neurofibrillary tangles, known as tau tangles, have been shown to prevent the transport of crucial molecules inside neurons. While, the senile plaques are accumulations of the β -amyloid ($A\beta$) fragments, which are thought to interfere with neural synapsis and contribute to neuronal cell death [1]. Understanding the molecular mechanisms around the formation and accumulation of $A\beta$ fragments and its effects on the pathology of AD has been of great importance for the research community over the years.

$A\beta$ accumulations are found in the brains of AD patients as oligomers, which are formed after proteolytic cleavage of the amyloid precursor protein (APP) by α -secretase, β -secretase, and a complex of γ -secretase enzymes [3,4]. Among the peptides derived from APP, $A\beta_{40}$ and $A\beta_{42}$ are associated with AD pathology, of which $A\beta_{42}$ has been found to be insoluble and deleterious, while $A\beta_{40}$ is soluble and most commonly found in AD and healthy brains (Figure 1) [5]. Since it is thought that $A\beta$ accumulation is a major cause for the loss of synapses that leads to the loss of memory in AD patients, studies inhibiting both $A\beta$ and APP have been widely

performed. However, drug treatments targeting APP and γ -secretase to reduce or eliminate A β production have shown to have deleterious effects including, alteration of cognition and emotion, and deterioration in hippocampal synaptic plasticity and memory [6]. Such findings highlight the importance of the molecular mechanisms underlying APP and A β for the maintenance of the healthy brain and point out the need for further studies of the roles that these proteins play.

Much attention has been given to studies focusing on the molecular mechanisms of A β and its function on both, healthy and AD brains. Thus, it would be beneficial to first evaluate the role of its precursor protein to understand the reasons behind A β accumulation in the AD brain. Studies *in vitro* and *in vivo* of AD have inhibited APP to investigate the effects that this protein has on the healthy and diseased brains. Previous research has indicated that, among many roles, APP is involved in maintenance of dendritic spine structures [7], modulation of microglia phenotype, inflammatory response, cell-cell adhesion [8], neural stem cell differentiation and migration, and adult neurogenesis [9]. These findings led to the conclusion that APP proteolytic products, especially A β , are found in large quantities in the diseased brains due to the function of APP in maintaining and developing the central nervous system (CNS).

The role of APP on adult neurogenesis and its contribution to regeneration after cell damage are of great interest for this study. Neurogenesis has been postulated as a novel therapeutic approach to treating neurodegenerative diseases [10]. Previous research has revealed the importance of APP in the proliferation of neural progenitor cells; and how dysfunction of APP could not only contribute to the aggregation of toxic A β peptides, but also lead to the impaired hippocampal neurogenesis related to AD [9,11, 12]. Considering that APP has a specific role in neurogenesis, evaluating its significance in the repair process of damaged neural

tissue can also justify why APP and its proteolytic products are found in great abundance in the AD brain. A β deposition and APP upregulation have been linked to pathological conditions, such as traumatic brain injury (TBI), where tissue damage in specific regions of the brain also results in inflammation, which is also common in AD [13]. IL-1 β , an inflammatory cytokine found in TBI, was shown to be produced in glial cells surrounded by amyloid plaques in AD [14], indicating that regulation of APP expression when TBI is present was associated with IL-1 β [13]. This implies that APP may be upregulated or overexpressed when neural tissue damage, leading to neuronal loss, is present.

The function that APP may have in neurogenesis and inflammatory responses suggests that APP production upon neural tissue damage may have a key role in the proliferation and migration of neural progenitors for tissue damage repair. Thus, this study aims to understand how APP influences wound healing after neural damage and disruption of neural cell connections. Neural progenitors (NP) derived from induced pluripotent stem cells (iPS) will be used as a *in vitro* model for both, the AD and the healthy brain (Control), for later comparison of the expression of APP in both conditions. iPS cells represent a novel model for neurodegenerative diseases, such as AD, because they maintain genotypic characteristics of the disease [3]. Thus, iPS cells allow for the study of AD by using any type of the patient's somatic cells, which will become pluripotent and later specialized into a targeted cell for pathological studies. The NPs-iPS cells will be exposed to a scratch assay that will mimic tissue damage by disrupting cell connections. Migration and proliferation processes will be evaluated as well as neural progenitor cell specialization to further understand how APP influences all these mechanisms in the AD.

BACKGROUND

The development of the nervous system and its regeneration are relevant for therapies involving neurodegenerative diseases [10]. In the past, it was thought that the nervous tissue could not be regenerated after birth, however the development of sophisticated techniques made it possible to locate areas in the brain where neural regeneration happens [9, 15]. Neurogenesis, in the adult mammalian brain comprises three areas, the subventricular zone, the olfactory bulb, and the hippocampus [10]. Adult neurogenesis requires NSCs proliferation, differentiation, and migration [9] to areas of the brain that require high plasticity [10]. The functional significance of adult neurogenesis remains unknown, nonetheless its importance has been linked to learning and memory processes [12].

In AD, hippocampal adult neurogenesis has been shown to be altered, but whether or not such alteration is positive or negative is still controversial. On one hand, impaired hippocampal adult neurogenesis has been linked to impairment of learning, memory, and cognitive functions in AD [12, 16]. Early impairment of adult neurogenesis is thought to enhance the progression of the disease [18]. Other studies have shown that hippocampal adult neurogenesis is increased in AD as a mechanism to partially compensate for the loss of neurons [17]. This could be because internal factors may promote the regenerative and recovery process of the brain. This discrepancy arises because it has been shown that various molecular players involved in AD pathology, like APP, are also involved in the process of hippocampal neurogenesis (Figure 2) [18].

APP is a single-pass transmembrane protein that is rapidly metabolized in neurons [6]. APP is best known by its proteolytic products that highlight two distinctive pathways, non-amyloidogenic and amyloidogenic, which give rise to the A β ₄₀ and A β ₄₂ peptides associated with AD (Figure 1). Among multiple functions that have been proposed for APP, its role in embryonic and adult neurogenesis [12] is of great importance for further understanding of AD and development of treatments against this disease. Studies *in vivo* and *in vitro* have demonstrated the importance of APP in regulating human NSCs (hNSCs) migration and differentiation [9, 19], which is important for the neurogenesis process in the normal adult brain. Other studies have also postulated that APP has a function in neural progenitor cell migration and axonal growth after neuronal damage or injury in *Drosophila* [19, 20] and cell culture [21]. These findings suggest that when damage of neuronal cells happen in neurodegenerative diseases like AD, APP may serve a crucial role in the processes that aim for restauration of the normal brain function.

APP has also been a major target for drugs that aim to reduce the production of A β . Previous research has demonstrated that Phenserine, an inhibitor of cholinesterase, can reduce the levels of APP by posttranscriptional regulation and subsequently the secretion of its proteolytic products *in vivo* and *in vitro* [22]. Phenserine regulates APP through an iron element located in the 5' untranslated region of the APP mRNA [22]. Shaw et. al. (2001) suggested that for a reduction of APP of nearly 50%, a specific concentration of Phenserine must be added to cells, in that case human glioblastoma and astrocytoma, for a specific period of time [22]. Using Phenserine to reduce APP production will serve to understand the role of APP by altering the normal conditions in which the NP-IPS cells produce this protein. Cell functions and the

influence of agents like Phenserine can also be evaluated to see what secondary effects the reduction of APP may have.

Studies of neurodegenerative diseases have been limited by few human disease models, the reliability of human tissue [3], and ethical issues. Newer discoveries have allowed for somatic cells to be reprogramed into stem cells that will be later differentiated in any type of cell that they are orchestrated to. The reprogramed cells are referred as iPS cells and represent a novel technique to model and treat neurodegenerative diseases [3]. iPS cells are differentiated into neural stem cells *in vitro* by using specific signal molecules to replicate the natural processes that drive neurogenesis [23]. Of these approaches, the inhibition of SMAD pathways has shown to be sufficient for conversion of iPS cells into NSCs in adherent conditions [24]. iPS cells can be used to mimic neurodegenerative diseases *in vitro* because disease-specific genetic marks can be conserved [3, 25]. Thus, *in vitro* models of AD using iPS cells will allow to further study the mechanisms involved in the disease and can serve as a guide for techniques used in *in vivo* studies or clinical trials.

Scratch assays allow for *in vitro* studies of regeneration as well as the agents involved. Axonal out-growth has been previously studied upon injury to evaluate the factors related to axonal regrowth and connectivity, and also indicated that scratch assays can be used to evaluate gliosis and astrocyte changes as well as axonal regrowth [25]. The efficiency of the scratch assay in studies involving neural cells suggests that it can be used as a model for evaluating the response that such cells have upon changes of factors that are key to regeneration processes. In this study, by addition of Phenserine, which may downregulate APP on NP – iPS cells.

OBJECTIVES

1. To differentiate Alzheimer's patient specific NSC-iPS cells into neural progenitor cells
2. To model neural tissue damage of neural progenitor cells through a scratch assay and analyze cell migration, proliferation, and cell specialization using fluorescent microscopy techniques
3. To understand how APP influences neuroregeneration of neural progenitor cells after injury by analyzing APP concentration using RT-PCR and fluorescent microscopy

HYPOTHESIS

High expression of APP and its proteolytic products are seen as hallmarks for Alzheimer's disease indicating the possible role of APP in processes involving neurodegeneration. APP has been shown to play multiple roles regarding development and the maintenance of the normal functioning of the brain.

It is hypothesized that APP is highly expressed on Alzheimer's disease due to its role on cell migration, proliferation, and differentiation after neural tissue damage. The experiments to be done in this thesis are attended to address what the role of APP is in the Alzheimer's disease condition. Previous *in vivo* and *in vitro* studies have demonstrated the role of APP in both neurogenesis and neuroregeneration, which support the idea that APP plays an important role in regulation of processes involving healing.

A novel model using patient-specific neural progenitor cells derived from induced pluripotent stem cells is proposed to evaluate the role that APP may have in the human adult brain upon tissue damage. Based on the information reviewed for this study, a scratch assay has not yet been performed that can indicate how APP may affect cell migration, proliferation, and differentiation upon tissue damage *in vitro*. Thus, it has been proposed to evaluate the possible role of APP these cell mechanisms upon an *in vitro* neural tissue damage model.

METHODS

Neural Differentiation of iPS Cells through Dual SMAD Inhibition

The cells used for this study were neural stem cells (NSCs) derived from induced pluripotent stem (iPS) cell lines generated by the dual SMAD inhibition methodology proposed by Chambers [24]. iPS cell lineages CW0018 (Control) and CW0064 (familial AD) from MyCellProducts were expanded and later treated with SMAD signaling inhibitors, Noggin and SB431542, for neural conversion (Figure 4). The resultant cells were then frozen using Synth-a-Freeze™ medium (Gibco®) and stored at in liquid nitrogen.

NSCs-iPSCs Expansion & Differentiation into Neural Progenitors

Frozen Control and AD NSCs derived from iPS cells were thawed at 37°C inside a water bath for 1-2 minutes followed by quick passaging to T-75 suspension flasks containing 20mL of pre-incubated (37°C, 5% CO₂) hNSC Media (500 mL DMEM/F-12, 1X B-27, 0.2ng/mL EGF, 2.0ng/mL rhFGF, 0.5U/mL Herparin, 1X antibiotic). NSCs-iPS were maintained with 50% media change every 3-4 days and incubated at 37°C, 5% CO₂ to allow cells to grow into neurospheres. Every 1-2 weeks, neurospheres were mechanically dissociated and split/passaged to enhance NSCs-iPS proliferation and formation of new neurospheres. After three passages, NSCs-iPS were induced to differentiate using media containing DMEM F12, 10% FBS, 1% antibiotic (NT2 media). Cells were maintained by performing 50% media exchange every 5-7 days with NT2 media and incubated at 37°C, 5% CO₂ for two weeks. Following differentiation, all media was collected in 50 mL conical tubes, and adhered cells were lifted using Versene 1X (Gibco® Life Technologies). 4mL of

Versene 1X was added to the cells, incubated for 5 minutes at 37°C, 5% CO₂, and later neutralized using 4mL of NT2 media. The contents of the flasks were added to the 50mL tubes, and cells were centrifuged at 1,200 rpm for 5 minutes. Supernatant was removed, and pellet was dissociated in NT2 media for cell count. 20,000 cells per well were seeded on seven 12-well adherent plates treated with polystyrene (FALCON ®). Cells were maintained using NT2 media by 50% media change every 5 days and incubation at 37°C, 5% CO₂ until 100% confluency was reached.

Neural Progenitor Cell Labeling

Vybrant™ Dio (Thermo Fisher Scientific) cell labeling solution was added to neural progenitor (NP) cells obtained from differentiation of NSCs-iPS in order to track NP cells. A concentration of 5ul/mL of Vybrant Dio was added to each well for a final volume of 400uL. Cells were then incubated for one hour and 15 minutes at 37°C, 5% CO₂. Vybrant Dio was then removed followed by three washes with 1X Phosphate Buffered Saline (PBS) pH 7.4 to remove any residuals from the cells. New NT2 media was added and cells were incubated for 24 hours before drug treatment.

Phenserine Treatment for Amyloid Precursor Protein Reduction

Neural progenitor (NP) cells obtained from differentiation of NSCs-iPS with NT2 media were treated with Phenserine Tartrate to reduce expression of APP. Based on Shaw's [22] findings for APP reduction, a concentration of 50uM was used to test reduction of APP synthesis by about 50% in 16 hours. A 10,000uM stock solution was prepared using 0.1g of Phenserine Tartrate and 35 mL of molecular grade water. A 50uM Phenserine Tartrate

solution made from stock using NT2 media. Old NT2 media was removed, and the 50uM solution in the positive control cells to a final volume of 1mL per well. Incubation took place for a period of 16 hours at 37°C, 5% CO₂.

Scratch Assay

Immediately after Phenserine treatment for 16 hours, a vertical 1.5 mm scratch was performed using a 1 mL pipette tip. After this, the 50uM Phenserine Tartrate solution was removed and replaced with new fresh NT2 media. Cells were incubated at 37°C, 5% CO₂ for 5 days. In order to follow the neurogenesis process of the NPCs, pictures were taken at 2, 24, 48, and 120 hours using 20X magnification in a fluorescent microscope. Images were captured at each time point on a specific area towards the left side of the scratch.

Immunocytochemistry of Neural Progenitors Treated with Phenserine and Scratch Assay

Following 120 hours after the scratch assay, NP-iPS cells were fixed using 100% iced cold methanol for 15 minutes at -20°C and washed three times with 1X PBS. Cells were permeabilized and membrane was blocked using a blocking buffer (1X PBS, 5% normal donkey serum, 0.3% Triton™ X-100) for 60 minutes at room temperature followed by three washes with 1X PBS. Plates containing Vybrant™ Dio were treated with anti BIII-tubulin antibody (1:1000) (Thermo Fisher Scientific). For the other plates, half of them were treated with anti BIII-tubulin (1:000) and anti GFAP (ASTRO6) (1:200) (Thermo Fisher Scientific) antibodies, and the other half were treated with anti BIII-tubulin (1:1000) and anti APP-22C11 (2.5:1,000) (abcam) antibodies. The cells were incubated overnight at 4°C. Following incubation, cells with BIII-tubulin only were treated with donkey anti-rabbit IgG TRITC

(1:200). The rest of the cells were treated with donkey anti-rabbit IgG TRICT and donkey anti-mouse IgG FITC (1:200) (Jackson Immuno Research Laboratories, INC) and incubated at 4°C overnight. After three washes with 1X PBS, DAPI (100:900) was added to all the cells followed by a 1x PBS wash. Pictures were taken towards the left side of the scratch using 20X magnification in a fluorescent microscope.

RNA Isolation of Neural Progenitors Treated with Phenserine and Scratch Assay using TRIzol

Total RNA from the previously plated neural progenitor cells was extracted using TRIzol (ambion®). AD and Control cells from 48 hours and 120 hours after scratch assay and their respective controls were used to check for APP gene expression at two different time points. Cells were first detached from the tissue treated plates using Versene 1X (Gibco® Life Technologies) following the previous stated protocol, which was adapted to be suitable for the area of each well (250 uL/well to wash, and 500 uL/well to lift the cells). Once detached, cells were centrifuged at 12,000 rpm for 5 minutes. The supernatant was removed, and the cells were resuspended in 500uL of TRIzol, moved to 1.5 mL tubes, followed by an incubation period of five minutes at room temperature. Then, 100 uL of Chloroform (Fisher Scientific) were added to each tube, and cells were then vortexed for 15 seconds and incubated for three minutes at room temperature followed by a centrifugation of 12,000 rpm for five minutes at 4°C.

After centrifugation, three phases were formed in each tube, and the top phase (aqueous one) containing the RNA was isolated and placed in a different tube. The tubes containing the aqueous solution were later treated with 250 uL of Isopropanol, inverted multiple times,

incubated for 10 minutes at room temperature, and then centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was then carefully isolated, and the pellet was washed with 500 uL of ice cold 95% ethanol and centrifuged at 7,500 rpm for 5 minutes at 4°C. The ethanol was removed, the pellet was let dry for 10 minutes and resuspended in 30 uL of molecular grade water. The RNA was then checked for purity using the Nanodrop™ (Thermo Fisher Scientific) and later stored at -20°C.

RT-PCR of Neural Progenitors Treated with Phenserine and Scratch Assay

cDNA was constructed from total RNA using the 50 Reactions SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR kit (Thermo Fisher Scientific). Following cDNA extraction, purity and concentration was checked using the Nanodrop™ (Thermo Fisher Scientific). Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was then performed using 4 uL MgCl₂, 10 uL 5X GoTaq Flexi Buffer, 2 uL dNTPs, 1uL GoTaqG2 Flexi DNA polymerase, 27 uL molecular grade water, 2 uL of reverse and 2 uL of forward primer and 2 uL of template. APP, GFAP, and BIII were checked on each of the samples. The APP primer used was 147 bp (+) GTGTTCTTTGCAGAAGATGTG, (-) CTCCACCACACCATGATG), the GFAP primer used was 266 pb (+) 5'-GCAGAGATGATGGAGCTCAATGACC-3' (-) and the BIII-tubulin primer used was 239 bp (+) 5'-ATGAGGGAGATCGTGCACAT-3'(-) 5'-GCCCTGAGCGGACACTGT-3.' The annealing temperature for BIII-tubulin and GFAP was 59°C and for APP was 48°C. The samples ran for 35 cycles.

Following PCR, gel electrophoresis was performed to check for expression of GFAP, BIII-tubulin, and APP genes. A 3% agarose gel was used to perform electrophoresis at 85 volts. The

ladder used for this procedure was the GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific).

The resulting gel was then analyzed for gene expression.

RESULTS & DISCUSSION

NSCs-iPS Cell Expansion and Differentiation

NSCs-iPS cells were cultured for six weeks on suspension flasks. Passages varied between 1-2 passages every other week based on the confluency and/or differentiation stage of the NSCs. Interestingly, AD NSCs-iPS cells grew bigger neurospheres, but little amount of them were produced when compared to Control NSCs-iPS cells (Figure 5). This could be due to differences in cell density after thawing for which NSCs-iPS cells were not counted. Stress and improper handling of the NSCs-iPS cells resulted in some cells spontaneously differentiating, which was highlighted by cells sticking to the bottom of the flask (Figure 5, C). When this happened, the neurospheres in suspension were removed and placed in a different flask to continue growing apart from spontaneously differentiated cells. At the end of expansion, there were seven T-75 flasks containing NSCs-iPS from both AD (four flasks) and Control (three flasks), which were used for neural differentiation.

NSCs-iPS cells previously mentioned were differentiated on the T-75 flasks for two weeks using NT2 media. Cells were then plated at high density in seven 12-well tissue treated plates. High density plating was used in order to obtain a monolayer and proceed with evaluations proposed [25]. Cells were then maintained for two more weeks before treatment with Phenserine and scratch assay. It is of note that AD cells grew slower than Control cells, which could have resulted in variations in the density of the cells before scratch assay. If so, some wells might have more cells than others resulting in more cells of a specific condition migrating more than cells of other conditions.

Neural Progenitor Cell Migration and Proliferation

Phenserine was used at 50 μ M to reduce APP expression by about 50% before scratch assay. Thus, the role of APP in the processes required for neural tissue regeneration could be evaluated by comparing cell migration, proliferation, and differentiation on both conditions, with and without Phenserine treatment. When comparing the Phenserine treated (P+) and non-treated (P-) conditions, there was not statistical significance in the reduction of APP (Figure 9, C). This may be due to APP not being significantly reduced after treatment with 50 μ M Phenserine on both cell lines. Interestingly, the results suggest that APP may have been reduced more in Control cells than in AD cells, however further studies on *in vitro* APP reduction by Phenserine are required to confirm this.

Before Phenserine treatment, Vybrant™ Dio cell labeling fluorescent dye was used for analysis of cell migration. After addition of Vybrant™ Dio and Phenserine, the scratch assay was successfully performed and sufficient for signaling neural progenitors to repair the damaged area. Five different replicates of the scratch assay were made, and three were selected for quantification. Figure 6 indicates the tracking of cell migration and proliferation after 2, 24, 48, and 120 hrs. upon scratch assay, and the respective immunofluorescence emitted by the neural progenitor cells on different Phenserine conditions. There is statistical significance in the immunofluorescence emitted by cells 2 hrs. after scratch and the immunofluorescence detected from cell at 120hrs. Data on the scratch area at 120 hrs. shows similar fluorescence emission than non-scratch area, suggesting cells may be migrating and proliferating (Figure 6 & 7).

Cell proliferation 120 hrs. after scratch assay was examined by nuclei cell count. Nuclei were stained with DAPI and manual count of each nucleus was performed. The average cell count did not show any statistical significance. Nevertheless, the results on cell proliferation showed that AD cells treated with Phenserine emit the highest immunofluorescence compared to the other samples (Figure 6), while it represents the sample with the least amount of cell proliferation (Figure 7). Thus, it could be possible that neuronal progenitor cells from AD patients in which APP is reduced, may present less cell proliferation and more cell migration rates than AD cells non treated or Control cells. These trends suggest that APP could be needed as a regulator of proliferation, which may occur after migration in order to initiate monolayer-repair of the *in vitro* model of neural tissue damage.

Neural Progenitor Cell Differentiation

120 hrs. after the scratch assay, differentiation of neural progenitor cells was evaluated using anti BIII-tubulin antibody to detect neurons and anti glial fibrillary acidic protein (GFAP) antibody to detect glial cells after scratch was closed (Figure 8). The results do not indicate any statistical significance between the different cell conditions for any of the progenitor cell types, but it was shown that both, neurons and glia, are present 120 hrs. after neural tissue injury. Based on the data collected, BIII-tubulin seems to be highly expressed on Control cells compared to AD cells, while AD cells appear to have more positive immunostaining rates for GFAP. These findings could indicate that APP may have a role in neural progenitor cell differentiation towards neuronal cells in the healthy brain (Control) and towards glial cells in the diseased brain (AD). A similar effect was shown in samples without scratch; AD NP cells seemed to have less BIII-tubulin immunostaining signal than Control NP cells, which could be related to APP

upregulation. If these assumptions were correct, then it is possible that inhibition of APP by Phenserine enhances BIII-tubulin immunostaining. This could explain why APP levels are higher in the diseased condition, since APP may be driving glial cell differentiation for primary neural tissue repair (AD) and neuronal differentiation in the healthy brain (Control).

In this scratch model, the regeneration time was determined to be 120hrs. Thus, in order to analyze when and whether GFAP, BIII- tubulin, and APP genes are regulated, RT-PCR was performed at 48 hrs. (D2) and 120hrs (D5) after scratch (Figure 10). Both BIII-tubulin and APP genes are expressed on all cells at all conditions at the different time points. The bands on D5 are more intense for both genes than the bands on D2, which may indicate a higher gene expression after neural tissue has been repaired due to full regeneration of the scratch area. Nevertheless, RT-qPCR should be done on the samples to specifically quantify the gene expression of APP and neuronal associated markers after a scratch assay.

APP Expression and Neuronal Cells

Neurons express high levels of APP in AD, which is one of the hallmarks in AD research. The correlation between the amount of APP expressed in neurons is crucial to evaluate whether or not APP affects neuronal cells. Figure 9 shows the expression of APP and BIII-tubulin (neuronal marker) 120hrs. after scratch assay. Positive immunofluorescence for APP and BIII-tubulin appear to be consistent on most cell conditions. In AD and Control conditions without scratch, AD seems to have lower intensity of immunofluorescence response for BIII-tubulin and APP compared to Control. AD cells non-treated with Phenserine showed signs to have lower immunoreactivity to BIII-tubulin compared to Phenserine treated AD cells. However, there was

not a visible difference between the immunoreactivity of APP in the Phenserine treated and non-treated cells. This data is correlated with the electrophoresis results presented on Figure 10. Finally, for Control cells, BIII-tubulin and APP immunofluorescence signal appears higher in non-treated cells compared to Phenserine treated cells.

These results suggest that Phenserine may decrease the amount of APP on Control cells, but not on AD cells *in vitro*. Additionally, it could be inferred that the levels of APP are slightly correlated to the number of neurons because APP may regulate neuronal differentiation and migration after neural tissue damage. This could be due to BIII-tubulin expression and cell migration being lower when APP is not reduced by Phenserine. Additionally, APP could be involved in glial differentiation if it were to inhibit neuronal migration and differentiation after neural tissue damage. Nevertheless, all these suggestions should be further evaluated when APP can be significantly lowered by a specific Phenserine concentration in AD cells derived from iPS cells in an *in vitro* model.

Future Directions

Future studies may focus on quantifying gene expression for neuronal and glial cells related markers as well as APP to indicate a more specific correlation between the levels of APP and neuronal and glial cells. It is also important to optimize in this model a concentration of APP that can reduce the amount of APP significantly enough to have more consistent and significant results that can indicate whether or not the inhibition of APP will have a key effect on cell migration, proliferation, and differentiation, since this study used a concentration previously used on glioblastoma cells [22]. Furthermore, immunostaining of neural progenitor cells at earlier time points would be beneficial to analyze how APP affects the first hours of neuroregeneration *in vitro*.

Further research into the specific role of APP in neurons will also lead to understanding how its reduction will influence the functioning of the normal brain compared to the diseased one. Thus, using specific factors for neural differentiation is recommended. Additionally, the role of APP in glial differentiation could be further study by analyzing what specific glial progenitor cells may be affected by APP. Finally, using specific factors and culture conditions to better mimic the normal functioning of the brain will result in more accurate data to understand the process behind the role of APP.

FIGURES

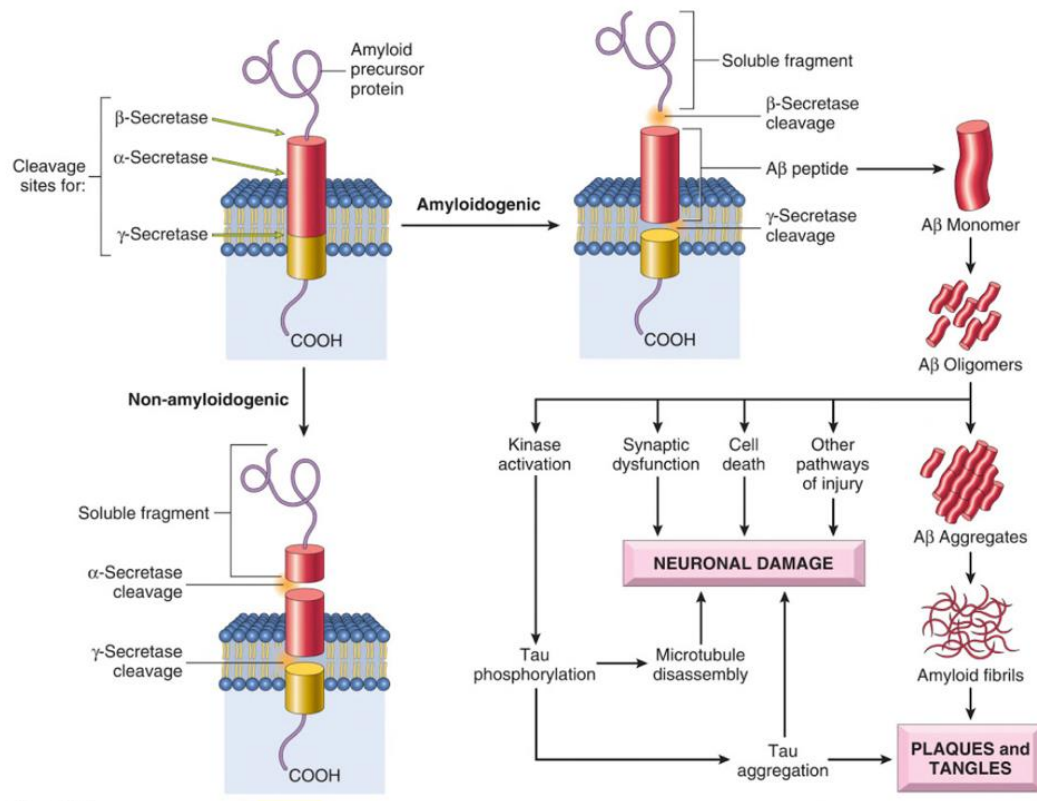


Figure 1. APP Proteolytic Cleavage in Alzheimer's Disease

The non-amyloidogenic pathway produces a soluble fragment upon cleavage by α -secretase at the N-terminus and γ -secretase at the C-terminus. Meanwhile, the amyloidogenic pathway results in the formation of a soluble fragment and the non-soluble A β protein upon the cleavage by β -secretase and γ -secretase. The A β accumulate and represent a hallmark for AD.

Source: *Robbins and Cotran Pathologic Basis of Disease, 9th Edition*

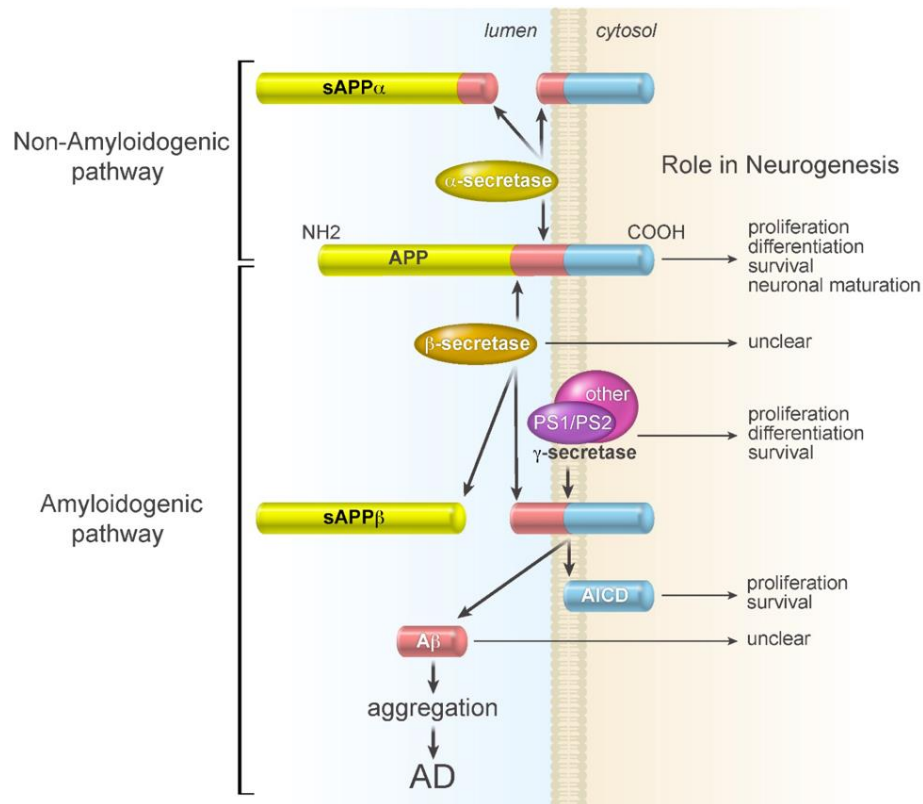


Figure 2. Alzheimer's Disease and Hippocampal Adult Neurogenesis

APP is thought to play important roles in the proliferation, differentiation, maturation, and survival of neural stem cells and neural progenitor cells in the adult hippocampus. APP is also a key hallmark for Alzheimer's disease as the precursor protein of A β oligomers. The amyloidogenic and non-amyloidogenic pathways for APP in Alzheimer's disease result in soluble APP (sAPP) and A β & AICD respectively upon the cleavage of secretases. Roles of the key players of Alzheimer's disease in hippocampal neurogenesis are summarized.

Source: <https://molecularneurodegeneration.biomedcentral.com/articles/10.1186/1750-1326-6-85>

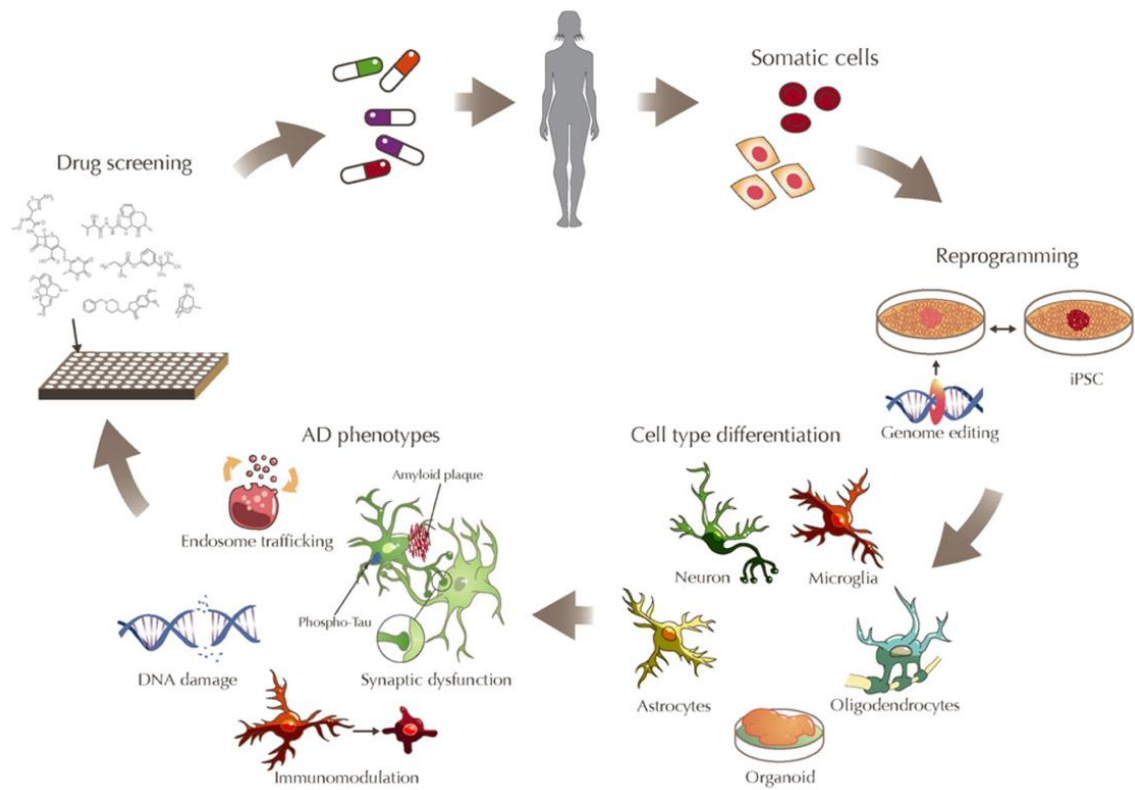
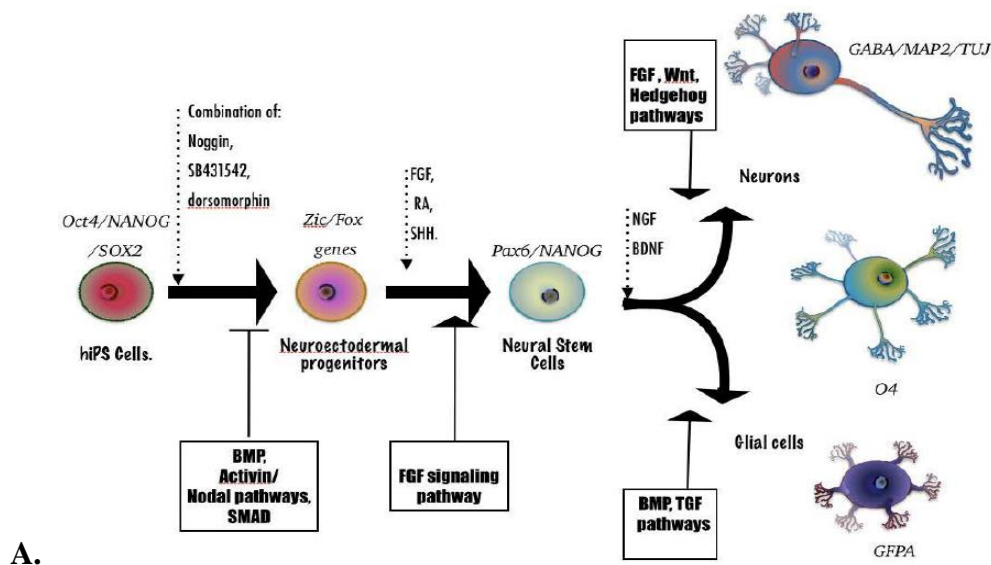


Figure 3. Alzheimer's Disease Modeled by iPS Cells

Patient specific somatic cells are reprogrammed using key transcription factors and signal molecules to be differentiated *in vitro* into neural progenitors. Resulting cells can be edited for mutations or transgenes, or these can be used to represent AD specific phenotypes to study the disease. At this point cells can also be used for drug screening or treatment with different molecules to target the disease.

Source: A.E. Mungenast et al. / Molecular and Cellular Neuroscience 73 (2016) 13–31



Source: <http://austinpublishinggroup.com/biomedical-engineering/fulltext/ajbe-v1-id1016.php>

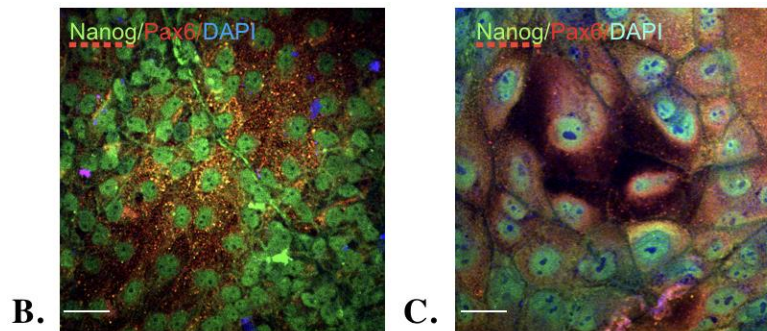


Figure 4. NSCs Obtained by Dual SMAD Inhibition

Human induced pluripotent stem (hiPS) cells are differentiated first into neuroectodermal progenitors and then into neural stem cells (NSCs) for future glial or neuronal differentiation. SMAD signaling is inhibited using Noggin, SB431542, or dorsomorphin that act as competitor of the BMP, activin, and nodal pluripotent cell pathways respectively. **A.** Pathway by which hiPS cells are differentiated into neuronal and glial cells. **B.** iPS cell lines 64 (AD) and **C.** 18 (Control) were induced to neural stem cells (NSCs) via dual SMAD inhibition using Noggin and SB431542 for neuroectodermal lineage to be activated. PAX 6 (neuroectodermal marker) and Nanog (stemness marker) are expressed indicating the existence of neural stem cells.



Figure 5. Neurospheres of NSCs-iPS

Neural stem cells derived from iPS cells (NSCs-iPS) were grown and maintained for six weeks using hNSC media. AD NSCs-iPS grew larger, but in smaller quantities, while Control NSCs-iPS neurospheres were smaller, but larger in quantity. **A.** AD neurosphere passage #3. **B.** Control neurosphere passage #3. **C.** Some cells may show spontaneous differentiation when cultured on NT2 media.

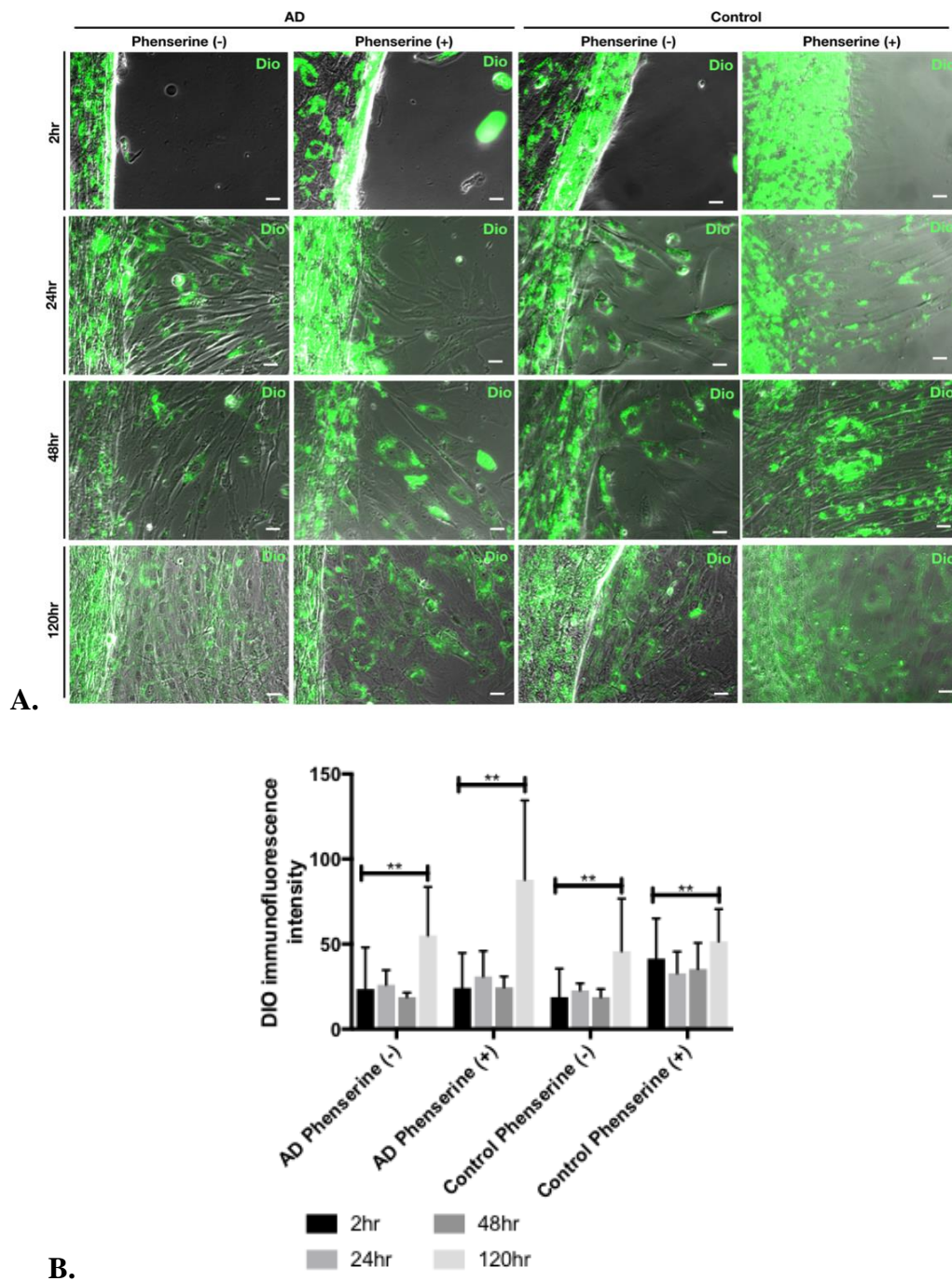


Figure 6. Neural Progenitor Cell Migration & Proliferation After Scratch Assay

A. Neural progenitor cells treated with Phenserine for 16hrs followed by a scratch assay. Dio was used to track migration and proliferation of NPCs at different time points (2hrs, 24hrs, 48hrs, and 120 hrs). **B.** Average immunofluorescence emitted by neural progenitor cells upon scratch assay at different time points.

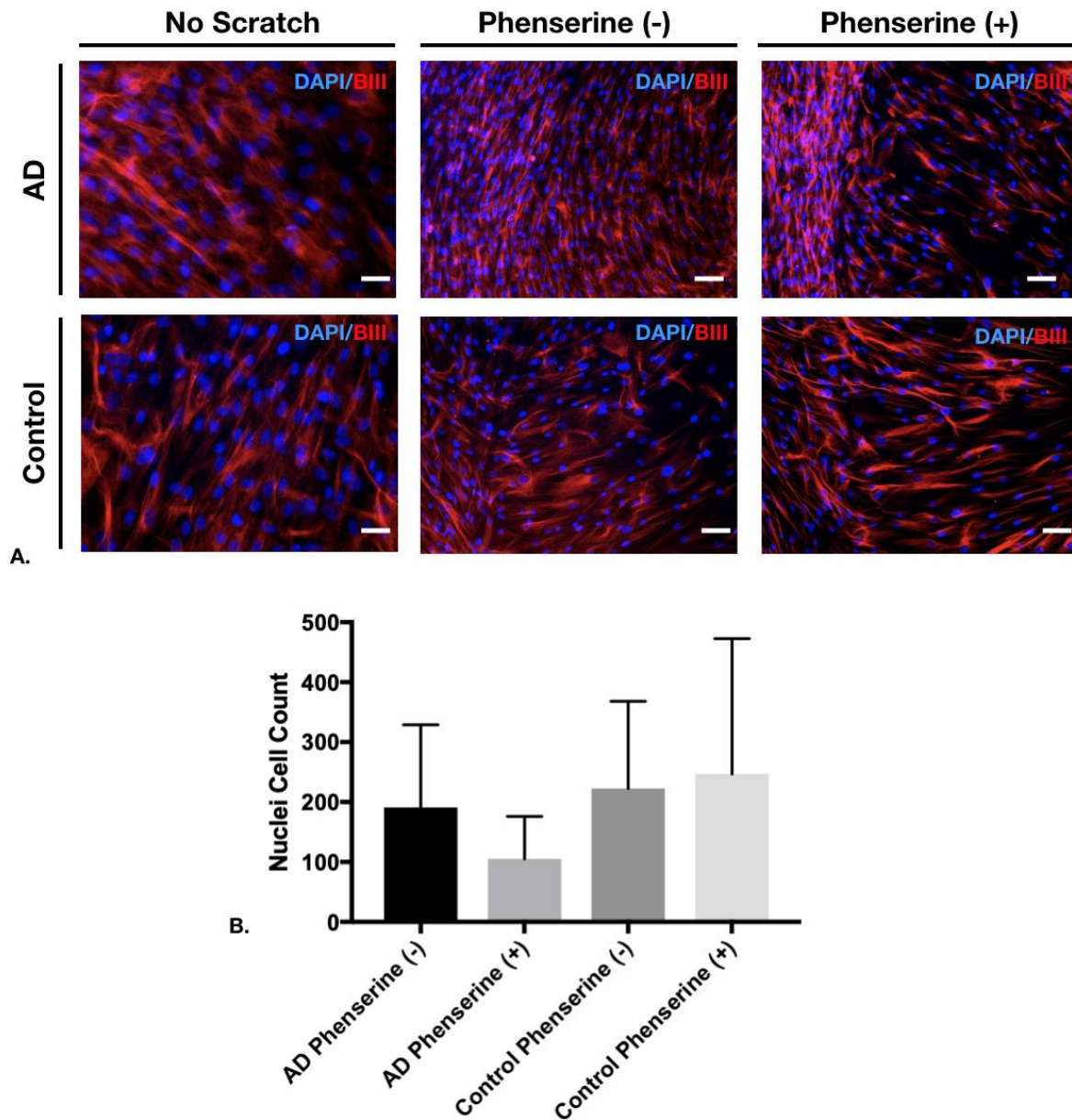


Figure 7: Neuronal Cell Expression and Cell Count

Neural cell proliferation 120hrs after scratch assay was recorded through cell nuclei count. **A.** BIII-tubulin (neuronal marker) expressed after scratch assay indicating neuronal cell proliferation from the area of scratch. **B.** Average number of cell nuclei in each neuronal cell condition.

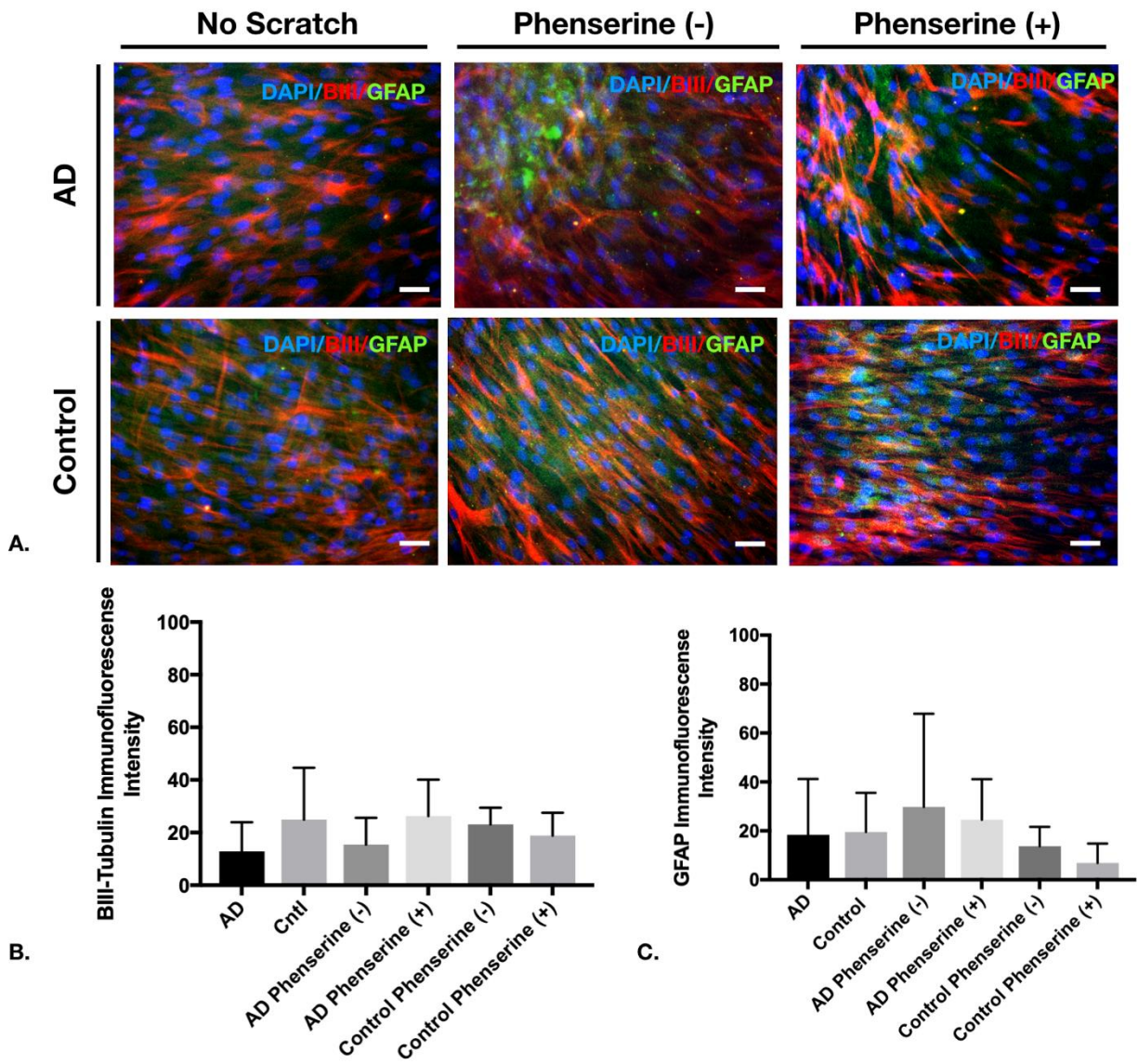


Figure 8. Neural Progenitor Cell Type Immunocytochemistry 120hrs after Scratch Assay

A. BIII-tubulin and GFAP were used to mark for neuronal and glial expression respectively on each control group 120 hrs. after scratch assay **B.** Immunofluorescence intensity for BIII-tubulin (neuronal marker) on neural progenitor cells 120hrs after scratch assay. **C.** GFAP (glial marker) immunofluorescence intensity for neural progenitor cells 120hrs after scratch assay.

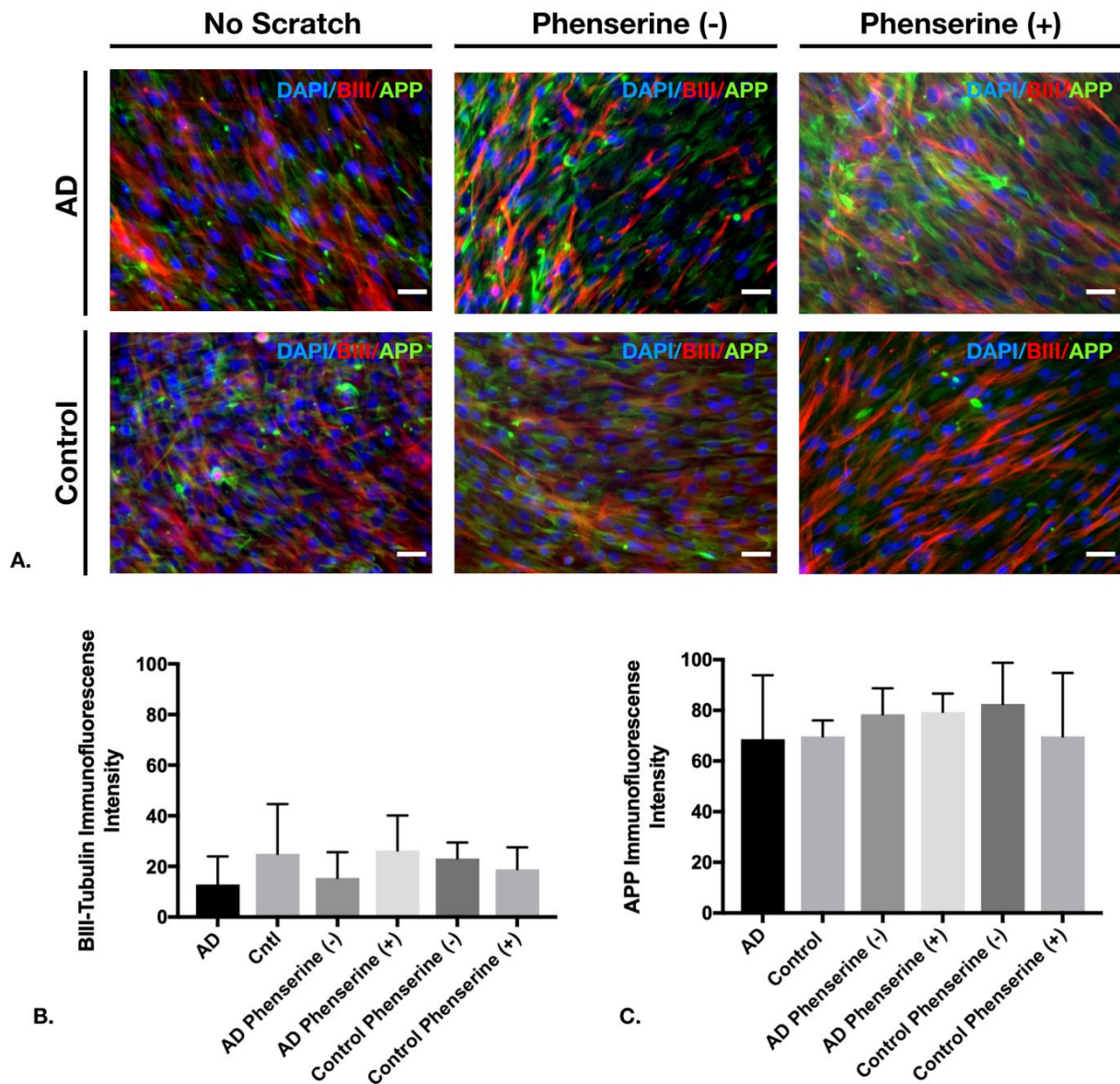


Figure 9: APP Expression in Neural Progenitor Cells 120hrs after Scratch Assay.

A. APP clone (22C11) and BIII-tubulin (neuronal marker) were used to indicate expression of APP and neurons on neural progenitor cells of all control groups 120hrs. after scratch assay. **B.** Immunofluorescence intensity for BIII-tubulin on neural progenitor cells 120 hrs. after scratch assay. **C.** APP immunofluorescence intensity 120 hrs. after scratch assay in neural progenitor cells.

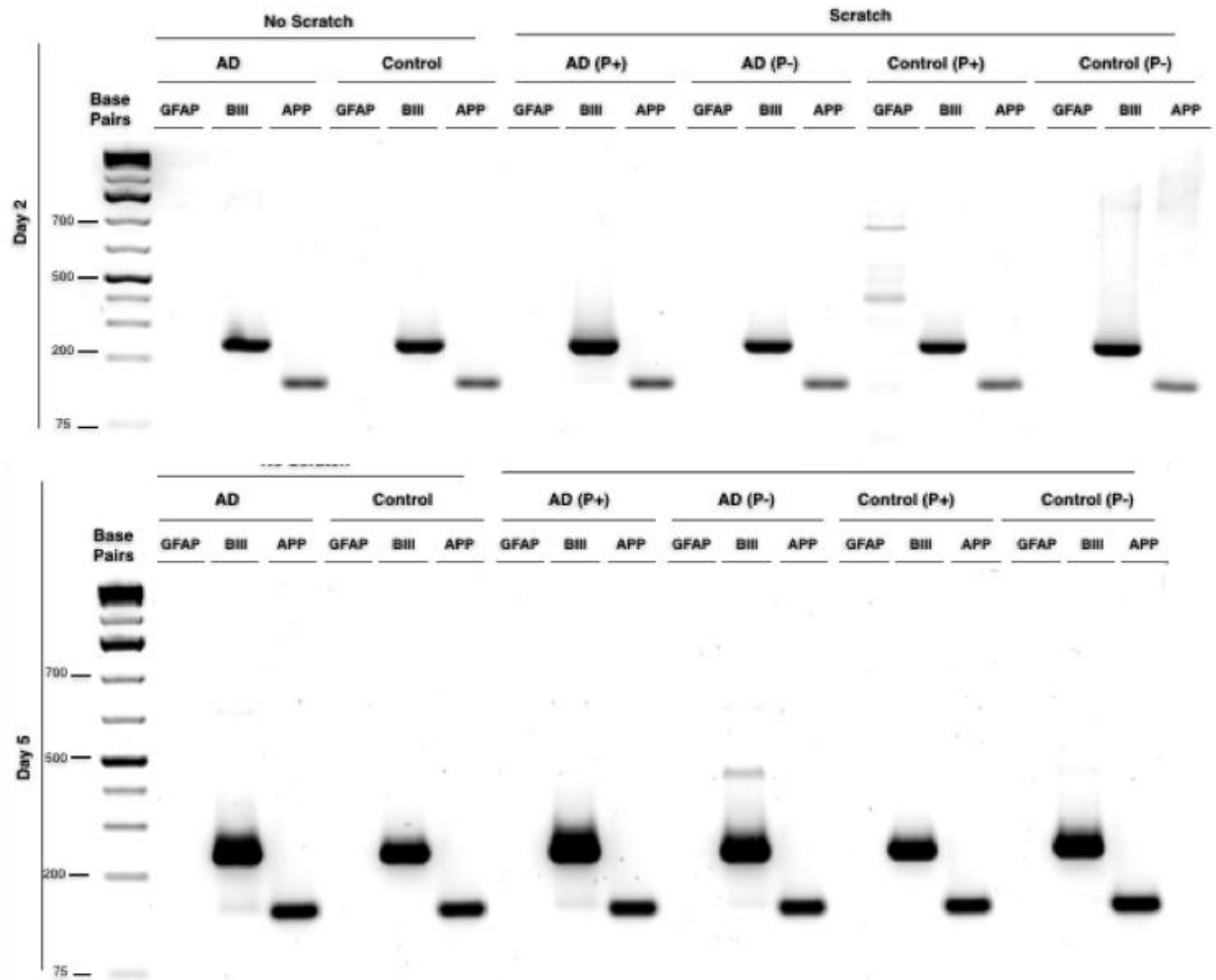


Figure 10: RT-PCR for BIII-tubulin, GFAP, and APP expression.

Gene expression for GFAP (glia), BIII-tubulin (neurons), and APP in neural progenitor cells on day 2 (48hrs) and day 5 (120hrs) after scratch assay. GFAP is 266 bp, BIII-tubulin is 239 bp, and APP is 147 bp long. Expression of both BIII-tubulin and APP can be detected on different conditions on both times (D2 & D5).

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