TRANSCRIPTION FACTOR REGULATION OF AMYLOID-BETA PATHWAY GENES BY SP1-MODULATING COMPOUNDS: A NOVEL APPROACH IN ALZHEIMER'S DISEASE

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Baindu L. Bayon

TRANSCRIPTION FACTOR REGULATION OF AMYLOID-BETA PATHWAY GENES BY SP1-MODULATING COMPOUNDS: A NOVEL APPROACH IN ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of neuritic plaques consisting of extracellular amyloid-beta (Aβ) and neurofibrillary tangles comprised of hyperphosphorylated microtubule associated tau. Aβ is produced following the cleavage of amyloid precursor protein (APP) by the enzyme BACE1. Transcription factors (TFs) are proteins involved in the regulation of gene transcription. Expression levels of some TFs are perturbed in AD. SP1 binding sites on both the APP and BACE1 promoters implicate its potential role in AD. Aβ peptide itself mediates activation of cyclin-dependent kinase 5 (CDK5), an enzyme which phosphorylates the FOXO (Forkhead Box) TFs. In order to study mechanisms of TF regulation of Aβ production in human models, neuronally differentiated cells as well as a primary human neurosphere culture were used to test the effects of TF-modulating compounds. Our hypothesis is that by targeting relevant TFs via pharmacological inhibitors in human cells, BACE1 activity or APP expression will decrease and Aβ production will be reduced as a result.

To test the involvement of TFs in the regulation of APP, we treated several mammalian cells lines and post-mitotic human neuronal cells with roscovitine, mithramycin A (MTM), MTM analogs (MTM-SDK, MTM-SK), and tolfenamic acid

(TA). MTM and TA treatment of neurons differentially activated several TFs implicated in AD. Treatment of differentiated neurospheres with MTM led to a significant decrease in APP and SP1 expression along with Aβ40 levels. Epigenetic mechanisms involve alteration of the binding affinity between DNA and transcription factors. We predict that modulation of these TFs may be influenced by epigenetic modifications. To test the effects of drugs on epigenetic markers, histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activity was measured. MTM-SDK significantly decreased DNMT activity in differentiated neuroblastoma cells, this may enhance or decrease the ability of SP1 to bind to target DNA and affect transcription of BACE1 or APP.

Targeting TF activity is a novel means to manipulate the amyloid pathway. Compounds modifying TF binding to sites on the BACE1 or APP promoters may provide a means to limit the production of amyloid-beta and slow the symptoms of AD.

Debomoy K. Lahiri, Ph.D., Chair

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ADNI	Alzheimer's disease neuroimaging initiative
AIDS	Acquired immune deficiency syndrome
APLP1	APP-like protein 1
APLP2	APP-like protein 2
APP	Amyloid precursor protein
ATF3	Cyclic AMP-dependent transcription factor
ATP	Adenosine triphosphate
Aβ	Amyloid β
BACE1	β-amyloid cleaving enzyme 1
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CDK5	Cyclin-dependent kinase 5
CHOP	CCAAT-enhancer-binding protein homologous protein
COX	Cyclooxygenase
c-PARP	c-Poly (ADP-ribose) polymerase
CTG	Cell Titer-Glo
DIV	Day in vitro
DMD	DNA binding domain
DMSO	Dimethyl sulfoxide
dNB	Differentiated neuroblastoma cells

DNMT	DNA methyltransferase
dNSPc	Differentiated neurosphere cells
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EOFAD	Early-onset familial Alzheimer’s disease
ERC	Entorhinal cortex
EWS-FLI1	Friend leukemia virus integration 1
FDA	Food and Drug Administration
FITC	Fluorescine isothiocyanate
GWAS	Genome wide association study
HD	Huntington’s disease
HDAC	Histone deacetylase
HEK293T	Human embryonic kidney cell line
HFN	Human fetal neurons
HRP	Horseradish peroxidase
ICC	Immunocytochemistry
iPSC	Induced pluripotent stem cell
IVIg	Intravenous immunoglobulins
LDH	Lactate dehydrogenase
LEARn	Latent early-life associated regulation
LPS	Lipopolysaccharide

LRP	Lipoprotein receptor-related protein
Mcl-1	Myeloid cell leukemia 1
MDR1	Multi-drug resistance efflux pump
MEM	Minimum essential media
miRNA	microRNA
MTM	Mithramycin A
MTM-SDK	Mithramycin analog SDK
MTM-SK	Mithramycin analog SK
mtmW	Gene encoding last acting enzyme in MTM synthetic pathway
$Na_v\beta_2$	Voltage gated sodium channel β_2 subunit
NB	Neuroblastoma cells
NBRA	Neuroblastoma cells differentiated in retinoic acid
NFκB	Nuclear factor kappa B
NGF	Neuronal growth factor
NRG1	Neuregulin 1
NRG3	Neuregulin 3
NSE	Neuron specific enolase
NSPc	Neurosphere cells
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
Pb	Lead
PBS	Phosphate buffered saline
PC12	Rat pheochromocytoma cell line

PET	positron emission tomography
PKS	Polyketide synthase
PSGL-1	P-selectin glycoprotein ligand 1
PVDF	polyvinylidene difluoride
RIPA	Radioimmunoprecipitation assay
RLU	Relative luminescence (light) units
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
SDS	Sodium dodecyl sulfate
SNAP25	Synaptiosomal associated protein 25
SNP	Single nucleotide polymorphism
SP3	Specificity protein 3
STAT1	Signal transducer and activator of transcription
TA	Tolfenamic acid
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween-20
TF	Transcription factor
TNF-α	Tumor necrosis factor alpha
undNSPc	Undifferentiated neurosphere cells
UTR	Untranslated region

I. Introduction

Alzheimer's disease (AD) is a major cause of death in the United States. The ways by which BACE1 is regulated likely plays a significant role in the way levels of amyloid-beta (Aβ) in the brain are controlled and therefore the symptoms of AD. BACE1 activity could play a critical role in the development of AD and may be an excellent drug target. Currently available BACE1 inhibiting drugs have been unsuccessful due to their many off-target effects. Widely used neuroscience models for AD study include cells derived from rodent or malignant sources. We aim to focus on not simply globally blocking activity of BACE1, but on controlling how the expression of BACE1 and APP is regulated by transcription factors in dividing and non-dividing cells, including a novel primary human brain model, differentiated neurosphere culture. Although it is known that SP1 is essential in the regulation of BACE1, the precise mechanism is not clear. It is imperative that the molecular mechanism related to transcription factor-modulated reduction of amyloid pathway genes be understood in order to manipulate these effects on Aβ production. Aβ peptide itself mediates activation of cyclin-dependent kinase 5 (CDK5), which phosphorylates the FOXO transcriptions factors. We believe that SP1 and CDK5 dysregulation are key players in the production of Aβ. *Our hypothesis is that by targeting relevant transcription factors via both gene silencing and pharmacological inhibitors in human cells, BACE1 activity or APP expression will decrease and Aβ production will be reduced as a result.* The rationale for these studies is that this work will provide evidence for transcription factors as possible mechanistic targets for AD

study while also providing evidence for the value of a new cell culture model for the study of neurodegenerative diseases as a whole.

A. Alzheimer's disease & the amyloid hypothesis

Alzheimer's disease (AD) is the most common form of dementia in the elderly population of the United States [1]. AD is a progressive, neurodegenerative disorder characterized by loss of cognition and memory, personality changes, decline in physical capacity, and eventually leads to loss of life [2]. More Americans are surviving into their 80s and 90s, which has led to an increased interest among scientists to investigate diseases seen in the elderly as well as elucidating better treatments and potential cures. The segment of the American population over 65 is expected to grow drastically with the "baby boomer generation" having entered this stage in 2011. Longer life expectancies and the aging baby boomers will dramatically increase the number of individuals at the highest risk of developing AD by 13 million people [3]. AD is currently the 6th-leading cause of death in the United States [4]. It is difficult to accurately determine the number of deaths that are caused by AD because of the way the cause of death may have been recorded. There is still a "blurred distinction between death *with* dementia and death *from* dementia [5]." It is likely that AD is a contributing factor in the deaths of more patients than is actually reported by the Center for Disease Control. Many patients with AD die from the disease itself or from conditions which AD caused, such as pneumonia. Deaths attributed to AD increased 68% between 2000 and 2010, which is more than those attributed to heart disease [6]. The progression of AD can be quite variable among

patients. Some patients over 65 survive four to eight years after diagnosis, while others may live up to 20 years with AD [7-10]. This is consistent with the slow, latent nature of this disease. This insidious characteristic of AD is particularly the cause of the public health burden of the disease. Most of the time patients spend in the severe stages of the disease require round-the-clock care, often in a nursing home or by unpaid caregivers [11]. The death rates for AD increase dramatically with age, which reflects the lack of effective treatments or a cure for this devastating illness.

AD pathology is believed to result from the multiple factors including genes, the environment, as well as epigenetic mechanisms. The major pathological hallmarks of AD are extracellular amyloid plaques made up of fibrillar amyloid-β (Aβ) peptide and intracellular neurofibrillary tangles made up of hyperphosphorylated tau [12]. Synaptic loss is also a key pathological finding that correlates with the cognitive impairment seen in AD [13]. Importantly, the dysregulation of the production of the 40-42 amino acid-long Aβ peptides have been identified as an underlying cause of the formation of senile plaques observed in the brains of AD patients. Histologically, these Aβ peptide plaques are most pronounced in the hippocampus and cerebral cortex of the brain which represent regions critical to AD pathology [14]. To date, the deposition of Aβ in senile plaques at autopsy represents an important diagnostic criterion for AD pathology. This project is aimed to address the hypothesis that the production of Aβ and its precursor (APP) can be modeled and studied *in vitro* in terms of neurobiology, transcription factor activation, and pharmacological effects.

i. The genetic basis of Alzheimer's disease

Early-onset AD (EOAD) develops before the age of 65 and late-onset AD (LOAD; sporadic AD) develops after 65 years [15]. EOAD is caused by dominant mutations in APP, PSEN1, and PSEN2. Sporadic AD has a genetic component of up to 60%-80%, however progression, disease severity, and onset are highly influenced by environmental factors as well [16].

ii. Amyloid plaque formation pathways

The rate-limiting step in the production of Aβ is the processing of amyloid-β precursor protein (APP) by a β-secretase, specifically β-site APP-cleaving enzyme (BACE1). Understanding the regulatory processes that modulate APP expression due to its direct role in the production of the precursor Aβ peptide is a key aim in proving the amyloid hypothesis. However, these regulatory mechanisms that affect APP production still remain unclear. According to the "amyloid hypothesis," AD is the result of the misregulation of the production or turnover of Aβ [17, 18]. There are mutations that may change how APP is cleaved to produce Aβ [19]. A key finding supporting the hypothesis is that most cases of EOAD are due to an increased ratio of the more toxic Aβ42 than Aβ40 peptide [19, 20]. There is increased Aβ42 production when there are autosomal dominant mutations in the genes for APP, presenilin 1 (PSEN1), and presenilin 2 (PSEN2) in EOFAD [21]. Furthermore, the late-onset AD risk factor, *APOE-ε4*, influences both Aβ clearance and its cerebral oligomerization [22]. In addition, individuals with Down's syndrome with an increased dosage of the APP gene located on chromosome 21 overproduce Aβ42 and develop EOFAD [23].

Extracellular Aβ42 is known to be toxic to synapses and neurites by reducing synaptic plasticity and altering synaptic function in AD [24-26].

iii. BACE1 dysregulation in Alzheimer's disease

One of the initial steps in the production of Aβ is the processing of APP by the aspartic protease BACE1. APP is a type I membrane protein that is processed by three proteases (α-, β-, and γ-secretase) in two distinct pathways that are both part of normal processing: the amyloidogenic and the non-amyloidogenic pathway. Cleavage of APP by BACE1 at the N-terminus of the Aβ-domain yields secreted sAPPβ and a C-terminal fragment of APP (C99). C99 then is further processed by γ-secretase which leads to Aβ peptide secretion and generation of the C-terminal fragment (CTF) of APP [27]. This is the amyloidogenic pathway and the focus of this work (Figure 1). . Most γ-secretase cleavage occurs after residue 40, resulting in Aβ40 peptide. Less commonly, however, cleavage occurs after residue 42. This Aβ42 peptide is more hydrophobic and has a higher tendency to aggregate than Aβ40, which makes it the key component of amyloid plaques [28].

Mice lacking BACE1 or γ-secretase do not produce Aβ or have cognitive deficits, making both secretases attractive drug targets [29-31]. Although BACE1 is expressed ubiquitously, there are higher expression levels detected in neurons that have increased BACE1 activity [32]. BACE1 exhibits tissue-specific expression with the highest levels in the pancreas, then brain [33]. Brain BACE1 expression is most profound in the hippocampus, a region known to have high expression of APP and Aβ. Interestingly, BACE1 mRNA and protein levels can

also be detected in brain areas which lack APP and almost never produce Aβ [34, 35]. This may indicate other functions of BACE1 in other parts of the brain. BACE1 also has other transmembrane protein substrates besides APP including low-density lipoprotein receptor-related protein (LRP), β-amyloid precursor-like protein-1 (APLP1), β-amyloid precursor-like protein 2 (APLP2), the voltage-gated sodium channel β_2 subunit ($Na_v\beta_2$), STGGal I, P-selectin glycoprotein ligand-1 (PSGL-1), neuregulin-1(NRG1) and neuregulin-3 (NRG3) [36-44]. Understanding these substrates and their functions is critical for understanding potential toxicity that could arise from inhibiting BACE1. BACE1 mRNA and protein levels remain stable in human brain during aging, but BACE1 enzymatic activity is increased [45]. BACE1 protein levels and activity are both elevated in AD brain [46-48]. This increased BACE1 activity in AD brain correlates with reduced α-secretase activity [49]. BACE1 also is responsible for maintaining the balance of astrogenesis and neurogenesis in the hippocampus [50].

In rodents, the deletion of BACE1 prevents the production of Aβ and reduces AD-related symptoms [31]. However, the changes seen in BACE1 null mice are associated with subtle and transient defects that are actually restored later on in development [51]. These findings suggest that these behavioral effects are mostly significant during development, but not during mature stages of life [52]. Therefore, in addition to a pathological endpoint of AD, BACE1 regulation likely represents an attractive drug target for treatment of AD pathogenesis. BACE1 is chiefly an attractive drug target because it is the rate-limiting enzyme for Aβ production in cells [32]. There is also genetic evidence for

the role BACE1 plays in AD. The Swedish double mutation responsible for a familial form of AD is located very close to the BACE1 cleavage site [28]. BACE1 also cleaves this Swedish mutant APP 10- to 100-fold more efficiently than wild-type APP [53, 54]. The 5' UTR and the promoter region of BACE1 contain many transcription factor binding sites, such as nuclear factor (NF)-κB, signal transducer and activator of transcription (STAT1), and specificity protein 1 (SP1) [55, 56]. These regulatory elements and sites for regulation by transcription factors allow for further examination into how the BACE1 promoter specifically is involved in AD pathogenesis. The BACE1 gene is located on chromosome 11q.23.3. It contains 9 exons and eight introns from a 30.6 kb region [57]. A 4.1 kb promoter sequence (rising crosshatch) and a 5' UTR (horizontal bars) are also present (Figure 2). BACE1 expression is high in neuronal cells and has been well correlated with Aβ production. Development of techniques to better understand the complex regulatory unit of the BACE1 gene and inhibit BACE1 activity could be essential in reducing levels of Aβ and slowing AD pathogenesis.

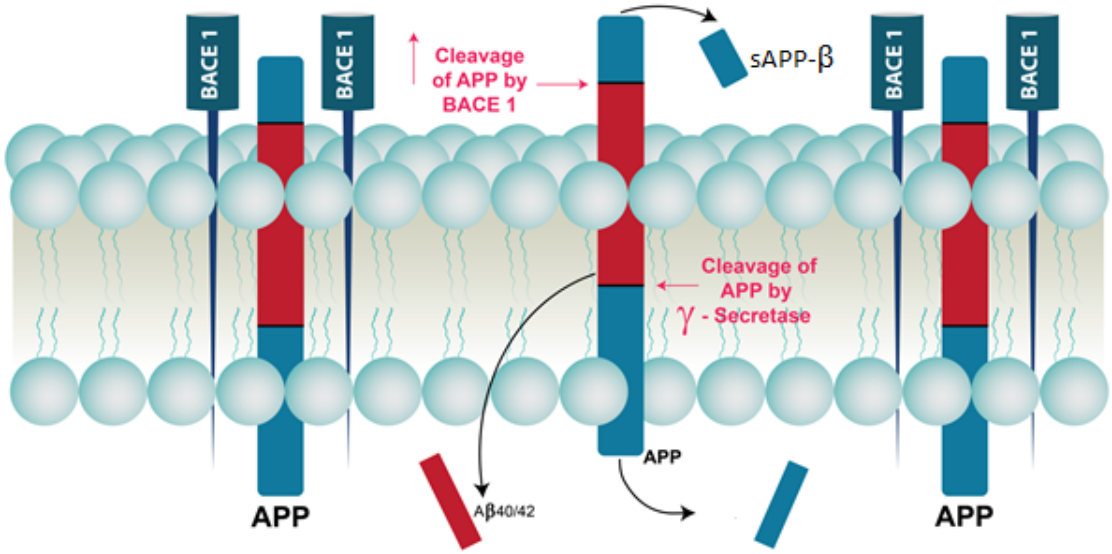


Figure 1. Amyloid-beta production in AD. The amyloid precursor protein (APP) is a transmembrane protein of 695, 751, or 770 amino acids depending on alternative splicing. The most understood post-translational modification of APP is proteolytic cleavage by secretases, and in the production of Aβ, these secretases are BACE1 and γ-secretase. BACE1 cleaves APP at the β-secretase site. Source: Bayon, B.L. *in preparation*.

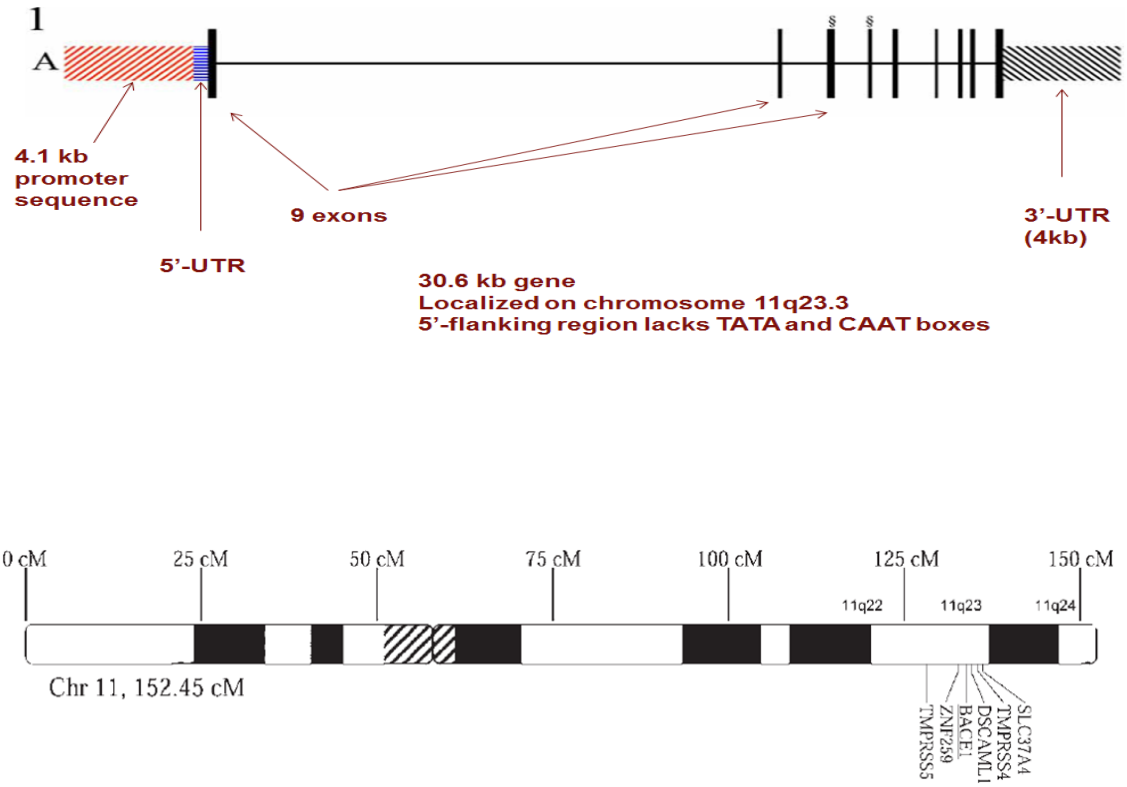


Figure 2. Scale Schematic of BACE1 Genomic Sequence. Located on chromosome 11q23. Indicating a 4.1 kb promoter sequence (rising cross-hatch), a 5'-UTR (horizontal bars), nine exons (vertical lines), eight introns (horizontal line), and a 3'-UTR/terminator region of up to 4 kb (falling cross-hatch). Lahiri et. al. 2006. J. Mol. Neurosci. 29; 65-80. Lahiri et. al. 2006. Curr. Alzheimer Res. 3(5): 475-483.

iv. APP dysregulation in Alzheimer's disease

Since its identification in 1987 by independent laboratories, many *APP* mutations have been identified that are associated with familial AD [58]. For instance, duplication or triplication of *APP* gene (seen in trisomy 21) leads to early-onset AD. Additionally, *APP*-deficient mice do not show notable changes other than decreased body mass, increased weakness of the extremities with increasing age, and gliosis of the brain [59].

v. CDK5 dysregulation in Alzheimer's disease

Aβ peptide itself mediates activation of cyclin-dependent kinase 5 (CDK5) which is activated and localized to the cell membrane under physiological conditions [60]. CDK5 has been implicated in brain development, neuronal survival, learning and memory formation [61]. It is known that CDK5 is dysregulated in AD [62]. Aβ may over-activate CDK5, which can result in reactivation of the cell cycle and apoptosis in post-mitotic neurons [60]. CDK5 activity can also result in phosphorylation of the tau protein [63]. The forkhead transcription factors (FOXO) have been implicated in many cellular processes. It has been demonstrated that FOXO1 phosphorylation is lost in CDK5-deficient post-mitotic neurons [64]. CDK5 directly phosphorylates FOXO3 in the brain, leading to increased nuclear translocation of FOXO3 [65]. Activated FOXO3 increases Aβ42 levels, which shows a relationship between phosphorylation of FOXO3 by CDK5 and AD [65]. This activation of the FOXO TFs Aβ could be targeted to treat AD.

B. Treatment of Alzheimer's disease

i. Current available treatments of Alzheimer's disease

There are three major therapeutic targets in the amyloid pathway to manipulate Aβ production: reduction of APP production, BACE1 inhibition, and γ-secretase inhibition. However, most of the treatments currently available for AD mostly aim to treat the late-stage symptoms of the disease. There are no therapies which prevent or will delay the age of onset of AD [66]. Of all treatments yet developed for AD, none significantly change the course of the disease. No drugs are available which target the APP processing pathway and Aβ production [28]. Many therapies are based on the idea that the cholinergic neurons undergo substantial progressive degeneration in AD [67, 68]. The development of cholinesterase inhibitors has been the focus of many pharmaceutical companies and in fact some have been approved by the US Food and Drug Administration (FDA) including donepezil, rivastigmine, and galantamine [69]. These therapies however are associated with small improvements in the symptoms associated with this devastating disease. This has led to the need for alternative strategies for potential treatments. An example of a new strategy is the targeting of microRNA (miRNA) to modulate AD-related gene expression [70]. It has been shown that miR-101 reduces APP levels in human cells [70]. Drug delivery is a key hurdle as there is not yet a delivery system that could get miRNAs to the cell population of interest. Strides are being made by adding specific chemical modifications such as conjugation of lipid moieties to miRNA molecules to target the liver [71], but not yet to the brain.

Drug development failures include the non-steroidal anti-inflammatory agent (NSAID) tarenflurbil, and acetylcholinesterase inhibitors metrifonate and phenserine [72]. Semagacestat, a γ-secretase inhibitor, had to be pulled during Phase III trials due to side effects as well [73]. Another γ-secretase inhibitor, Avagacestat, made it to Phase II trials before being terminated due to clinical worsening [74]. From these studies, it seems focusing on a γ-secretase inhibition is unlikely to result in a viable drug treatment for AD. Recently, the failure of Eli Lilly's β-site APP-cleaving enzyme (BACE1) targeting drug LY2886721 made national headlines for its off-target effects on liver function [75]. However, it is not clear whether this toxicity was directly caused by BACE1 inhibition [75]. Most recently, the November 2016 failure of solanezumab in phase 3 trials, cast further doubt on the amyloid hypothesis. This drug, a monoclonal antibody, was designed to promote clearance of Aβ [76] and slow the progression of the disease [77]. There is a need to identify new drug targets in the preclinical setting, which may require reevaluation of current treatment agents or development of new model systems.

ii. Treatment modalities under development in Alzheimer's disease

Emerging therapies include Aβ removal by immunization or intravenous immunoglobulins (IVIg), decreasing Aβ production, blocking Aβ signaling, and blocking tau-mediated toxicity [69]. Transcription factors themselves can be difficult drug targets as they can be involved in the regulation of several genes. However, modulating a transcription factor such as SP1 with tissue-specific

expression in the brain could produce a moderate decrease in BACE1 activity that could benefit AD patients in combination with other therapies.

C. Transcription factors as druggable targets

Transcription factors (TFs) as so-called “druggable” targets has been long debated. The idea that a drug may regulate several genes by changing their activity may lead to many unwanted side effects. TFs bind to specific sequences present within promoters, enhancers, and other regulatory regions of DNA, which is negatively charged. This means that TFs tend to be very positively charged and compounds targeting them would need to be negatively charged. Charged molecules do not easily cross cellular membranes, which makes them less than ideal for clinical practice. There are, however, several advantages of targeting transcription factors in lieu of more conventional drug targets. Specificity protein 1 (SP1) is a transcription factor (TF) expressed in brain and involved in neuronal survival that may be dysregulated in Alzheimer’s disease (AD). We aim to propose the continued study of SP1 as a transcription factor to target in AD. SP1, SP1-modulating drugs, their structures, and their effect of genes of the amyloid pathway are discussed in the following sections.

i. Role of SP1 in Alzheimer’s disease

The BACE1 promoter contains a number of transcription factor binding sites [55]. SP1 is a human transcription factor that binds to G-C rich motifs and can act as a repressor or as an activator. SP1 regulates the transcription of a number of genes involved in a number of processes. To better understand the mechanism of BACE1 gene expression and the relationship to SP1, Christensen

et. al. examined several cis-acting elements in the 5' flanking region [78]. They demonstrated via transcriptional activation and gel shift assays that the BACE1 promoter contains a functional SP1 response element. They were also able to show that overexpression of Sp1 potentiates BACE1 expression and therefore generation of Aβ (Figure 3). The BACE1 gene promoter was cloned and the transcription start site was mapped using a 5' genome walking strategy and a primer extension assay. Sequence analysis showed that the BACE1 gene had a complex transcriptional unit with a possible SP1 site. This region, when deleted, showed marked BACE1 gene promoter activity. Gel shift assays were performed to confirm whether or not this region was an SP1 site or not. The gel shift assay showed the presence of an SP1 binding element at bp-911. Deletion of this binding element from the BACE1 gene promoter resulted in a significant decrease in luciferase activity in a human embryonic kidney cell line (HEK293T). They performed experiments in a rat pheochromocytoma cell line (PC12), and deletion of the SP1 binding site again reduced the promoter activity as was seen in HEK293T. The SP1-binding inhibitor mithramycin A (MTM) was used to treat cells since it has been shown to inhibit SP1 binding to DNA. Treatment with MTM for 48 hours resulted in a significant reduction in BACE1 promoter activity in both a dose and time dependent manner (Figure 3). They were also able to show that SP1 plays a critical role in the transcriptional regulation of the BACE1 gene in humans by using SP1-/- ES cells (from SP1-KO mice). Activation of BACE1 promoter activity was markedly reduced compared to that of wild type. This observation also demonstrated that SP1 is required for the BACE1 gene to

be properly transcribed. To discover whether this requirement has an effect on APP processing and Aβ production, levels of BACE1, APP C99 (the major BACE1 cleavage product) and Aβ were analyzed in stably APP695-transfected HEK293 cells. This stable cell line was then transfected with SP1 cDNA and then cells were treated with MTM. MTM drastically reduced the generation of APP, C99, and Aβ. These results suggest that inhibition of SP1 by MTM down regulates BACE1 expression and that BACE1 is a downstream target of SP1.

Basha et al. were able to induce a robust and long-lasting increase in SP1 expression in rats exposed to lead during the prenatal stage [79]. This protracted induction resulted in an increase in APP expression and Aβ production thus linking amyloidogenesis and SP1. Studies outlined in this work look to expand on these findings by investigating mechanism of the effect SP1 inhibition of BACE1 expression has in human neurons.

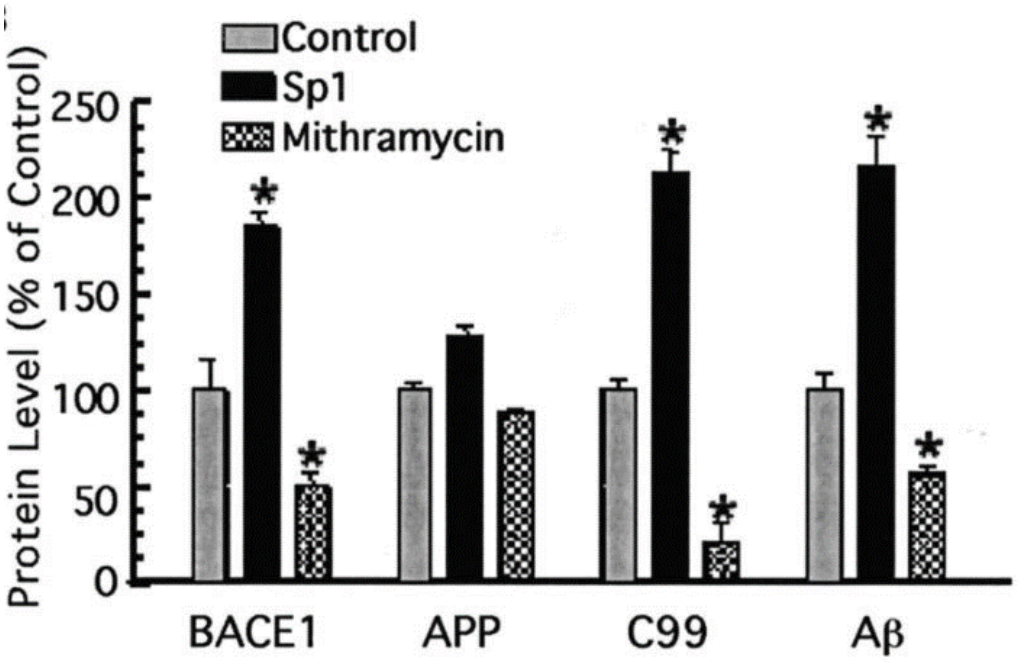


Figure 3. Overexpression of SP1 in HEK293T cells increases BACE1 and Aβ; treatment with an SP1 inhibitor decreases BACE1 and Aβ. Christensen MA, Zhou W, Qing H, Lehman A, Philipsen S, Song W. 2004. Transcriptional regulation of bace1, the beta-amyloid precursor protein beta-secretase, by SP1. Molecular and cellular biology 24:865-874.

ii. The latent early-life regulation model: An epigenetic framework for transcription factor regulation of late-onset neurodegenerative disease

The latent early-life associated regulation (LEARn) model provides specific mechanisms to predict targets for late-onset disorders such as AD [46]. This model is based on the environmental induction of dormant epigenetic changes [47]. LEARn explains idiopathic disorders on the basis of accumulation of 'hits' throughout life. Hits may be environmental, genetic or epigenetic [80]. After accumulating additional environmental insults, disease develops later in life. Proteins that are susceptible to this phenomenon can be called "LEARned [48]" This theory proposes that a first hit occurs early in life [47]. This could simply be an exposure to an environmental toxin, change in nutrition, or head trauma [49, 50, 53, 54]. This exposure leads to stress than causes a change in regulatory sequences of the gene at some important, developmental stage of life. This so-called hit is maintained epigenetically (i.e. methylation or oxidation of DNA) during a latency period for what could be many years [46]. During this period of time, normal gene expression is observed and there are no phenotypic manifestations of the first hit. Eventually, a second hit (i.e. exposure to metals, dietary factors) occurs and becomes an additional stressor. This causes the accelerated loss of function of this gene expression and the threshold of disease and symptom development is crossed. LEARn proposes a sort of intermediate step between genotype and phenotype. The environment induces this epigenetic modification of a gene to produce a "somatic epitype [54]." This somatic epitype

determines gene expression levels which correspond to the pathological state (Figure 4). Therefore, the LEARn model operates through the regulatory region or promoter of a gene. Basha et. al. demonstrated that rats exposed to lead (Pb) early in life showed a transient increase in APP expression, yet a delayed overexpression 20 months after exposure had ended [79]. This late increase in expression also correlated with an increase in SP1 activity. They also observed that APP expression, SP1 activity, and Aβ levels did not respond to Pb exposure later on in life. This suggests that these environmental influences that occur during development have a unique effect on amyloidogenesis later in life. They profiled a number of transcription factors after developmental exposure to Pb including SP1 by performing a macroarray screen on postnatal day 5. Postnatal day 5 is the day in rats that correlates with the highest APP levels. SP1 levels were highly induced following Pb exposure. Lifelong SP1 binding was then monitored. SP1 DNA-binding increased dramatically 20 months after Pb exposure had ceased. We propose that most AD cases would follow a LEARn etiology and specifically, that SP1 is a “LEARned” protein. Although these studies are critical and quite informative in discovering the connection between APP, SP1, Pb, and Aβ levels, they are limited as they do not address these interactions in a human model. Studies outlined in this work specifically address this limitation as some studies will be conducted in differentiated neurospheres derived from human fetal brain tissue.

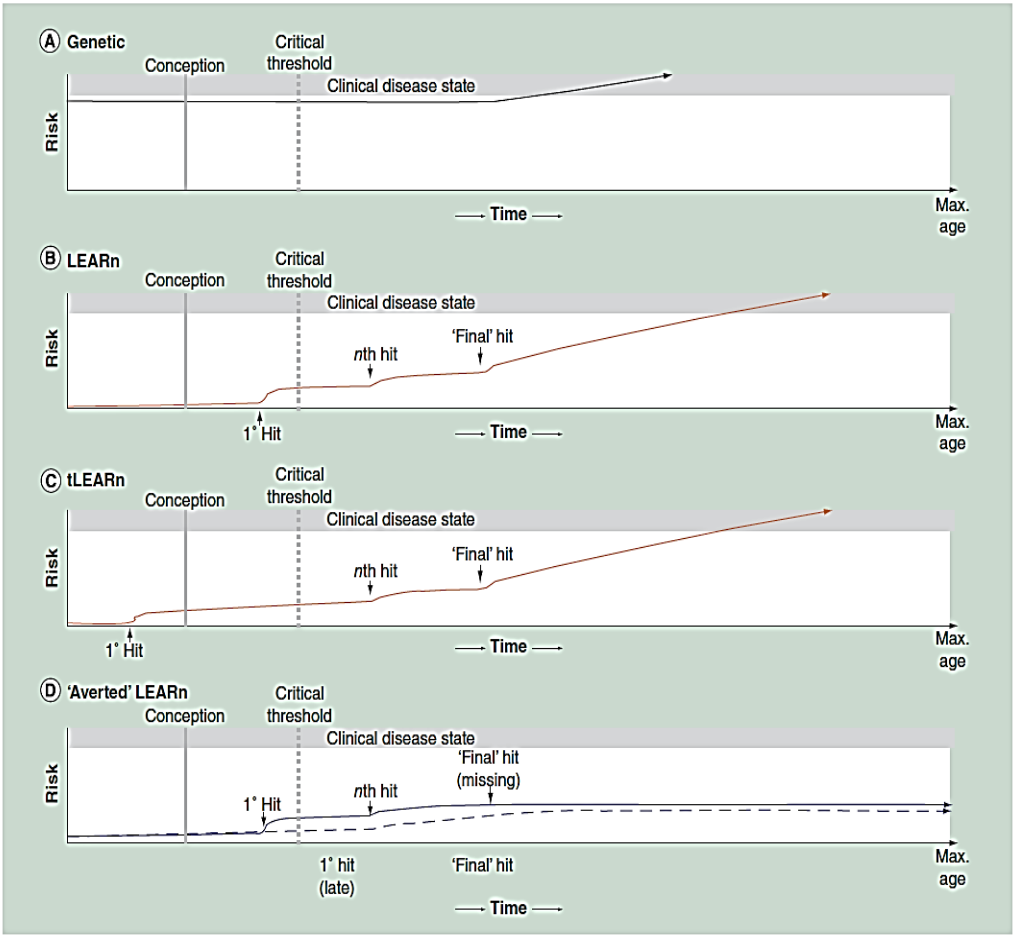


Figure 4. The latent early-life associated regulation model.

(A) Genetic pathway for a 'purely' genetic disorder, such as Alzheimer's disease (AD) due to familial dominant mutation. AD-associated genes' DNA sequence variant determines the disease state. **(B)** Development of disease via LEARn pathway. A primary 'hit' between conception and a critical developmental time point leads to latent epigenetic changes. If followed by subsequent 'hits', accumulated risk factor effects will reach clinical disease state as in the sporadic AD. **(C)** LEARn pathway across generations: t-LEARn. Similar to LEARn progression except the original 'hit' has occurs in an earlier generation and is transmitted asymptomatically by epigenetic inheritance. **(D)**

Remediation pathway or ‘Averted’ LEARn. Given that many epigenetic marker states can be altered by environmental factors, including nutrition and drugs, the possibility exists that one or more of the effects of a given ‘hit’ may be reversed by these means. Should this occur, accumulated effects will not reach clinical disease [80].

iii. Transcription factor modulating compounds

a. Mithramycin A

Mithramycin A (MTM) is a natural aureolic acid-type aromatic polykeditde antibiotic produced by various species of Streptomyces [81]. MTM binds GC-rich regions within the DNA minor groove and displaces SP1 family proteins from the binding sites of many genes [82]. MTM displaces SP1 from sites on the promoters of several oncogenes, which has made it an extensively studied chemotherapeutic agent for years [83]. By displacing SP1 from its binding sites on oncogene promoters, MTM induces apoptosis via down-regulation of *myeloid cell leukemia-1* (*Mcl-1*) [84]. Sleiman et al. were able to show via promoter analysis and chromatin immunoprecipitations that MTM does not displace SP1 or SP3 from every promoter in neurons, illustrating that MTM is able to selectively displace SP1 at some sites and not others [83]. MTM diffuses rapidly into neuroblastoma cells to bind to DNA [85]. The affinity MTM has for GC-rich sequences and its ability to compete with SP1 for binding to BACE1 promoter DNA are two factors influencing its pharmacological potential in AD. MTM is a tricyclic chromophore with a hydrophilic side chain at the 3-position [83]. Multiple acyl-CoA units proceed through type II polyketide synthase (PKS) mediated condensation to form demethylpremithramycinone and premithramycinone. Premithramycinone is glycosylated, then C-methylated to form premithramycin B. The fourth ring is cleaved, the 4'-keto group is decarboxylated and reduced catalyzed by oxygenase MtmOIV and ketoreductase MtmW [86]. MTM is FDA approved for treatment of testicular cancer and leukemia [83]. Other applications

for MTM use include Paget bone disease and hypercalcemia [87, 88]. MTM shows promise in the treatment of glioblastomas, inhibition of the Friend leukemia virus integration 1 (EWS-FLI1) transcription factor in pancreatic cancer, and in the inhibition of the multi-drug resistance efflux pump MDR1 [89-91]. Because MTM inhibits SP1 interaction with binding sites of SV40 and c-myc promoters, it also has a potential role in AIDS therapy [92]

A few studies have shown that MTM can enhance neuronal survival. Chatterjee et al. showed that MTM is able to suppress neuronal apoptosis as a result of DNA damage or oxidative stress while not affecting global protein synthesis [93]. MTM has been shown to improve behavior and increase survival of Huntington's disease (HD) mice [94, 95]. Studies also suggest that MTM can protect against dopaminergic neurotoxicity in mice after the administration of methamphetamine [96]. The protective effects MTM has in neurons may derive from a reduction in SP1 binding promoters of typically associated with cancer [83]. There is a reduction in the expression of these select oncogenes that "covaries with a biological phenotype of neuroprotection in neurons" [83]. Inhibition of Myc, c-Src, Erk, and Raf protects neurons [83, 97-101]. There may be a mechanistic connection between neurodegenerative and oncogenic pathways. Mutations in *APP* can not only lead to AD, but also leukemia [102]. The Cdc42/Rac and retinoblastoma p130 oncogenic pathways have also been shown to contribute to AD [103, 104]. If MTM is functioning in both oncogenic and neurodegenerative pathways, it will be interesting to elucidate the mechanistic pathway possibly related to aging. These studies suggest the

potential for the use of MTM as a therapeutic agent in CNS diseases with neurodegeneration. Use of MTM has been limited due to its side effects including gastrointestinal, bone marrow, hepatic, and renal toxicities [105-107].

b. MTM-SDK and MTM-SK

MTM analogs MTM-SDK and MTM-SK were created by genetic manipulation of the MTM biosynthetic pathway [108, 109]. MTM analogs have favorable characteristics such as enhanced potency and decreased toxicity to cells. MTM-SDK (for Short side chain, DiKeto) differs from MTM in structure and length of the hydrophilic side chain [83]. It has been previously shown that the 3-side chain is partially responsible for MTM interacting with the phosphate backbone of DNA [110, 111]. MTM-SDK has a shorter side chain at the 3-position as compared to MTM. MTM-SDK and MTM-SK (for Short side chain, Keto) have a greater ability to block SP1 binding to DNA than does MTM [85, 112]. This leads to the idea that the 3-side chain is critical in the activity of MTM and therefore its analogs. A genetically engineered *S. argillaceus* strain, M7W1, contains an inactivated mtmW gene. The mtmW gene encodes MtmW, the last acting enzyme in the MTM biosynthetic pathway [113]. The inactivation of this gene produces the MTM analogs MTM-SDK and MTM-SK. MTM-SDK and MTM-SK are formed from the spontaneous rearrangement of MTM-DK [114]. Synthetic modifications allow for the introduction of different functions and properties of MTM analogs. Both MTM-SDK and MTM-SK exhibit higher anticancer activity than does MTM [85, 115]. Both analogs are being used in studies looking to improve bioavailability and increase plasma retention time

[114]. MTM-SK has been shown to exhibit the same potency as MTM in neuroblastoma cells [85].

c. Tolfenamic acid

Tolfenamic acid (TA) is a non-steroid anti-inflammatory drug (NSAID) which inhibits cyclooxygenase (COX) activity and synthesis of prostaglandin [116]. TA has also been shown to attenuate the degradation of SP1 which reduces the transcription of both APP and BACE1 [117, 118]. Expression of BACE1 is reduced as well as Aβ production [119, 120]. TA has also been shown to rescue neuroblastoma cells from an increase in SP1, APP, and Aβ induced by lead exposure [118]. The exact mechanism by which TA promotes degradation of SP1 is unknown. Intracellular neurofibrillary tau tangles are a hallmark in AD brain and in transgenic mice, and interestingly TA lowers both tau mRNA and protein [121]. TA has been implicated in the regulation of many tumor suppressive TFs such as CHOP and ATF3 [122]. Evidence suggests that TA blocks TNF-α- or LPS-induced NF-κB activation in human cells [123]. Caspases, survivin, c-Met, VEGF, c-PARP, and cyclin expression are all modulated by TA [124]. TA decreases the gene expression of CDK5, which is responsible for the phosphorylation of tau [125]. TA has been shown to interrupt the de novo synthesis of APP and to change downstream levels of Aβ in C57BL/6 mice and in hemizygous R1.40 transgenics [119, 120]. It is promising that TA reduces plaque pathology in AD mouse models along with improving memory [126]. These many interactions indicate that TA can affect apoptotic, anti-inflammatory, as well as Aβ pathways. This complex mechanism of action may allow TA to

partially mediate many effects, including the suppression of BACE1 promoter activity via SP1 degradation, while also interrupting the pathological tau tangle phosphorylation pathway leading to a promising use in AD studies. TA (brand name Clotam) has been used in Europe and Asia for the treatment of migraine headaches and rheumatoid arthritis for several decades [127, 128]. The safety profile and pharmacokinetics of TA in humans has already been well established with its long use for the treatment of migraine headaches in Europe. Its interference with SP1 has not revealed worrying effects on adults taking this drug [129]. However, the side effects of TA are consistent with other NSAIDs. Gastrointestinal bleeding and ulcers can occur at high doses, but occurs less frequently that with ketoprofen, indomethacin, and naproxen [130]. Chronic administration of TA in mice did not result in changes in hemoglobin or hematocrit [124].

d. Roscovitine

CDK5 has been implicated in AD. Mice with increased cerebral CDK5 activity were shown to have increased levels of BACE1 and Aβ [131, 132]. Also, CDK5 along with its activator proteins p25 and p35 have been shown to be dysregulated in post-mortem AD brain [133]. Forkhead box proteins are a transcriptional activator which triggers neuronal cell death upon oxidative stress (Brunet et al. 1999). FOXO1 and FOXO3a are highly expressed in the human brain, specifically in areas susceptible to AD (Hoekman et al. 2006). Dysregulated CDK5 causes neurotoxic Aβ processing and cell death, by phosphorylating FOXO3a (Shi et al. 2016).

Roscovitine is a selective cyclin-dependent kinase (CDK) inhibitor with various anti-inflammatory, -proliferative, -apoptotic, and neuroprotective effects in different cell types [134]. It is being researched for the treatment of non-small cell lung cancer (NSCLC), leukemia, HIV infection, and cystic fibrosis [135]. Roscovitine has been shown to interrupt activation of NF-κB and to reduce expression of TNF-α-induced proinflammatory gene [136]. It also downregulates Mcl-1 and decreases cyclin D1 expression [134, 137]. As a CDK5 inhibitor, treatment with roscovitine can influence phosphorylation of the FOXO proteins. CDK5 inhibition has been shown to stabilize FOXO1 leading to an increase in its transcriptional activity [138].

D. Research plan

In order to investigate the effect of TF-modulating drugs on other transcription factors and Aβ pathway genes, a well-characterized human cell culture model is needed. Given the expense, unknown variables present, and differences in the development of AD-pathology of AD model animals, tissue culture has been chosen as the model for this work. Tissue culture allows cells to provide information on cell response to transfection and drug treatment in a faster time frame than with model animals. Although further removed from disease states than animal models, we choose human models which are more aligned with the species specific characteristics of AD. Furthermore, our use of primary human neuronal (HFN) and differentiated neurospheres (dNSPc) allow a mixed culture with populations what recapitulate that of the brain. These cells are a better approximation of the *in vivo* cell populations than can be available in cancer cell lines. Fetal tissues will be used because they were obtainable in regular quantities and easy to dissociate, allowing for greater chances for data collection per shipment of tissue. Prior to drug treatments and manipulations via siRNA, it was necessary to characterize the cultures and optimize culture conditions. Human fetal neuron culture was previously described by our laboratory [139]. Characterization of both undifferentiated and differentiated neurosphere culture were done as part of this work in order to determine the nature of the cell population under particular cell culture conditions over time.

Cell lines were used in preliminary experiments to measure tolerance of cells to the drugs of interest, antibody characterization, and validation. It is important to understand the differences in protein expression across cell types.

Human epithelial (HeLa), glial (U373), neuroblastoma (SK-N-SH, NBRA), and induced pluripotent stem cells (iPSCs) were all used for these purposes. In addition, the rat pheochromocytoma cell line, PC12 was used to optimize plating conditions for the Incucyte Zoom. This is due to the ease of detection of neurites in PC12 differentiated with neuronal growth factor (NGF). It was presumed that in the primary cell cultures (HFN and NSPc), there would be more variation in protein expression. Use of well-characterized, pre- and post-mitotic cell lines provide a detailed understanding of the effect of MTM, MTM-SDK, MTM-SK, TA, and roscovitine on protein expression.

Many proteins have been implicated in AD. Of particular interest in this study is the aberrant expression of BACE1, APP, and SP1 (Figure 6). Also of interest is CDK5 and its role in activation of TFs FOXO1 and FOXO3. The proteins of interest in these cultures over time also include neuronal and glial markers NSE and GFAP, respectively. Synaptic proteins synaptophysin and SNAP25 are both considered because they have been shown to be reduced in AD [140]. Of greater interest, however, is how SP1, FOXO1, FOXO3, and other transcription factors regulate AD-relevant genes. Thus, understanding how these drugs, thought to be TF-modulators, potentially activate several other TFs that mediate the reduction of BACE1 activity, APP expression, and/or amyloid-beta production. This was carried out by the transduction of both primary cells and cell lines with reporter genes driven by TF activation followed by the appropriate reporter assay. These, along with predictive tools and literature searches, help to elucidate a possible mechanism for SP1 or other TF-modulation of Aβ pathway

genes. It is expected that SP1 along with a few other TFs modulate BACE1 activity, APP expression, and Aβ clearance/production in a manner that could be disease-modifying with the proper pharmacological intervention.

II. Materials and Resources

For a comprehensive list of reagents used in experiments, see Table 2.

A. Tissue culture

Cells were maintained and treated according to cell line type. HeLa, U373, and naïve SK-N-SH cells were cultured in MEM containing 10% fetal bovine serum (FBS) and 1X antibiotic. PC12 were cultured in RPMI medium containing 10% heat-inactivated horse serum and 5% FBS. Cells were seeded into tissue culture treated polystyrene multiwall plates (Corning).

MTM was obtained from Santa Cruz. Analogs MTM-SDK and MTM-SK were obtained from the laboratory of Rajiv Ratan of the Burke Medical Research Institute in Cornell University. TA was obtained from Sigma. All drugs were reconstituted in DMSO to make stock solutions that were diluted in media for treatments.

B. Western immunoblotting

Western immunoblotting was used in order to detect proteins of interest.

i. BSA protein estimation and sample preparation

Cells were lysed in RIPA buffer containing complete protease inhibitor cocktail. Protein content of these samples was estimated using the Pierce BSA Protein Assay Kit (Thermo). BSA was used to generate a standard curve to allow calculation of the protein content of each sample. This estimation allowed

sample volume to be adjusted and for relatively equal loading of protein per lane. Loading volume in the gels were limiting, and in some cases, the maximum volume loaded was dependent on the least concentrated sample. Samples were prepared in 4X Laemmli Sample Buffer (BIO-RAD) and heated to 95°C for 10 minutes using a thermal cycler (BIO-RAD).

ii. Electrophoresis conditions

Samples were loaded onto SDS-PAGE gels and 200 V was applied for the time needed to allow the bromophenol blue tracking dye front to pass completely through the gel (generally 1 h). The gels were then removed and transferred to membranes via wet or dry transfer methods.

iii. Criterion XT Western blot system

The Criterion XT Western Blot System (BIO-RAD) is a precast SDS-PAGE system. In these experiments, the 12% polyacrylamide, 26-lane gels were used. Large numbers of samples were able to be resolved on the same gel (e.g. drug treatments with $n \geq 4$ for each compound and dosage).

iv. Mini Protean II Western blot system

In experiments requiring a higher loading volume than the 26-lane gels allow, the Mimi Protean II System (BIO-RAD) was used. Western blots produced by this system allow 10-15 samples, but at volumes up to 35 µL as compared to about 12 µL in the Criterion XT gels.

v. Wet transfer system

Gels were removed and placed in a tray of transfer buffer containing 20% methanol, and allowed to equilibrate for 10 min. Proteins were then transferred

to a 0.22 µm pore size PDVF membrane (BIO-RAD). Protein transfer was visualized using 0.1% (w/v) Ponceau S stain in 5% v/v acetic acid (Fisher). Excess stain not bound to protein was washed off with 5% acetic acid and the blot was electronically scanned using a desktop scanner.

vi. iBlot gel transfer system

The Invitrogen iBlot® 7-Minute Blotting System is comprised of the iBlot™ Gel Transfer Device and iBlot™ Gel Transfer Stacks. An 8 min transfer protocol was used. Gel was placed on the transfer membrane of the anode stack and air bubbles were removed with a roller. One piece of water pre-soaked filter paper was placed on top of the gel. The cathode stack was placed on the filter paper and air bubbles were again removed with a roller. A disposable sponge was placed in the lid of the gel transfer device and the anode-filter paper-gel-cathode stack was placed on the blotting surface. The lid of the device was closed and latched, then the 8 minute program was selected (20 V). After transfer, the membrane was removed, quickly rinsed with methanol, then acetic acid before Ponceau S staining.

vii. Antibody incubation conditions

After Ponceau S stain was rinsed off with several washes of 1X Tris buffered saline (TBS; Thermo), primary antibodies were applied in a solution of casein blocker in TBS at antibody-specific dilutions listed in Table 1. Primary antibodies were applied overnight at 4°C. The following day, blots were washed 4 times for 5 minutes each in TBST before secondary antibody solution was added. The appropriate host-specific HRP secondary antibody was also

prepared in casein blocker in TBS. The blots were incubated in secondary antibody solution at room temperature for 1 h on a rocker. After incubation, the blots were washed 6 times for 5 minutes each in TBST.

viii. Detection

Protein was detected using enhanced chemiluminenscence (ECL; Thermo) according to manufacturer's instructions. The detection reagents were applied to the blot for 2 min, then the blot was placed in a transparent plastic sheet protector and exposed to film (Thermo) for the required amount of time specific to each antibody. Exposure times of between 30 sec to 3 min were required for most proteins depending on the relative abundance of the protein in a particular sample.

ix. Antibodies

Primary antibodies and source are listed in Table 1. Horseradish peroxidase (HRP) conjugated secondary antibodies were obtained from Pierce (Rockford, IL; anti-mouse and anti-goat) or from Sigma. For immunocytochemistry, secondary antibodies and avidin conjugates were all obtained from Jackson ImmunoResearch Laboratories (West Grove, PA).

a. SP1, APP, and BACE1 levels in dividing cells vs. nondividing cells

Antibodies were optimized for use in various cell types. Decreasing concentrations of proteins from cell lysates of HeLa, SK-N-SH, differentiated neurospheres, and U373 were loaded onto a 10% polyacrylamide gel and separated by SDS-PAGE. Protein was transferred to PVDF by iBlot gel transfer system and probed with SP1 antibody (Figure 5, 6). Expression levels of SP1

are lower in glial and neuroblastoma cell lines then in human neurosphere cells and HeLa. Specificity of the SP1 antibody was tested by SP1 siRNA transfection of HeLa cells (Figure 7b). SP1 siRNA transfection significantly knocked down SP1 protein levels in HeLa as detected by Western blot.

To optimize use of BACE1 antibody, 2 commercially available antibodies were tested which recognize different epitopes of BACE1 (Figure 9). Human adult brain, human adult AD brain, human fetal brain (DIV0), U373, SK-N-SH cells differentiated with retinoic acid (NBRA), HeLa, and rat kidney were analyzed via Western blot for levels of BACE1 (Figure 9). BACE1 levels were detected in fetal brain by both abcam and R&D antibodies. BACE1 is also easily detected in adult brain by the R&D antibody. Probing with R&D BACE1 antibody, however, did not reveal bands in kidney, U373, NBRA, or HeLa cell lysates. The abcam antibody was able to detect protein in adult brain, U373, NBRA, HeLa, and fetal brain, but not in kidney. BACE1 abcam antibody was chosen for use in future experiments. A cohort of human brain specimens from human cortex was obtained from the lab of Peter Nelson. The cohort is comprised of 5 controls and 15 AD specimens. The AD specimens are further sub-grouped by exposure to AD therapeutics (Figure 10). Brain specimens were analyzed for detection of BACE1 and housekeeping proteins. To validate the authenticity of the APP antibody, an siRNA experiment was performed in U373 (Figure 11).

Table 1. Antibodies used in Western immunoblotting (WB), immunocytochemistry (ICC), and flow cytometry (FC)

Antibody (Ab)	Source	Catalog	Host	Use	Ab Type	Expected Size (kDa)	Dilution
Alzheimer Precursor Protein (APP) A4	Millipore	MAB348-100UL	mouse	WB	1°	110,120,130	1:3000
BACE1	Vassar Lab		mouse	WB	1°	75	1:500
BACE1	abcam	ab2077	rabbit	WB	1°	70	1:500
BACE1	R&D	MAB931	mouse	WB	1°	60-75	1:1000
Biotin-SP-conjugated AffiniPure Donkey Anti-Mouse IgG	Jackson-Antibodies	715065-150	donkey	ICC, FC	2°		1:1000
CDK5	Cell Signaling	2506	rabbit	WB	1°	30	1:1000
Cy3-conjugated Donkey Anti-Goat	Jackson-Antibodies	705-165-147	donkey	ICC, FC	2°		1:300
Cy3-conjugated Donkey Anti-Rabbit	Jackson-Antibodies	711-165-152	donkey	ICC, FC	2°		1:300
Doublecortin	Cell Signaling	4604	rabbit	WB	2°		1:1000
DTAF-conjugated Streptavidin	Jackson-Antibodies	016-010-084		ICC, FC	2°		
Foxo1	Cell Signaling	2880	Rabbit	WB	1°	78-82	1:1000
Foxo3a	Cell Signaling	12829	Rabbit	WB	1°	82-97	1:1000
GAPDH	Sigma	G9545	rabbit	WB	1°	36	1:10,000
GFAP	Sigma	G9269	rabbit	WB, ICC, FC	1°	46	1:2000
Goat anti-Rabbit IgG Secondary Antibody, HRP conjugate	Thermo Scientific	31460	goat	ICC, FC	2°		1:3000

HRP Western Blot Anti-Mouse IgG	Rockland	KCB002	goat	WB	2°		1:200,000
Nestin	abcam	ab22035	mouse	WB, ICC	1°	176	1:3000
NSE	Abcam	ab16873	rabbit	WB, ICC, FC	1°	47	1:1000
Pan-Neuronal	Millipore	MAB2300	mouse	ICC, FC	1°		1:1000
PSD95	Neuromab	75-028	mouse	WB	1°	95-110	1:1000
SNAP25	Chemicon	MAB331	mouse	WB	1°	26-27	1:1000
SP1	Millipore	07-645	rabbit	WB	1°	95-105	1:500
Synaptophysin	Cell Signaling	5461	rabbit	WB	1°	38	1:1000
α-tubulin	Sigma-Aldrich	T9026	mouse	WB	1°	50	1:50,000
β-actin	Sigma-Aldrich	A5441	mouse	WB	1°	42	1:50,000

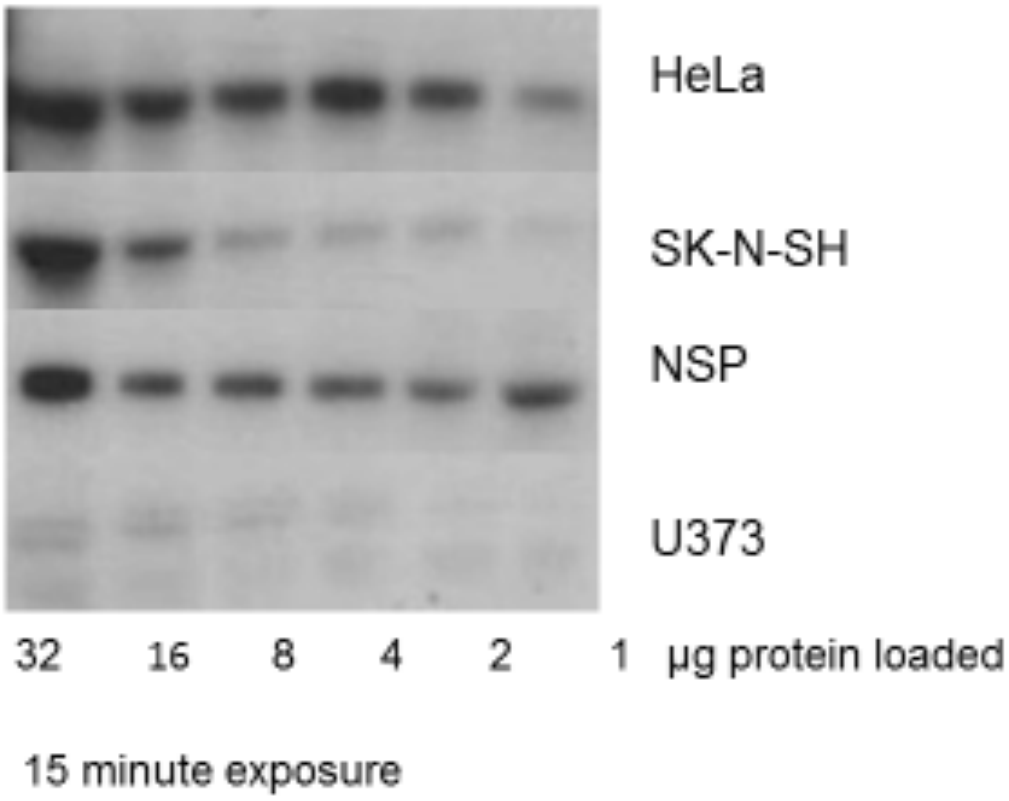


Figure 5. Differential expression of SP1 in various cell types. Decreasing concentrations of protein from HeLa, SK-N-SH, differentiated neurospheres (NSP), and U373 were loaded on a gel, transferred by iBlot to PVDF membrane, and probed with SP1 antibody. Signal is faint in U373 even at higher concentrations of lysate loaded. Expression levels of SP1 are noticeably lower in glial and neuroblastoma cell lines.

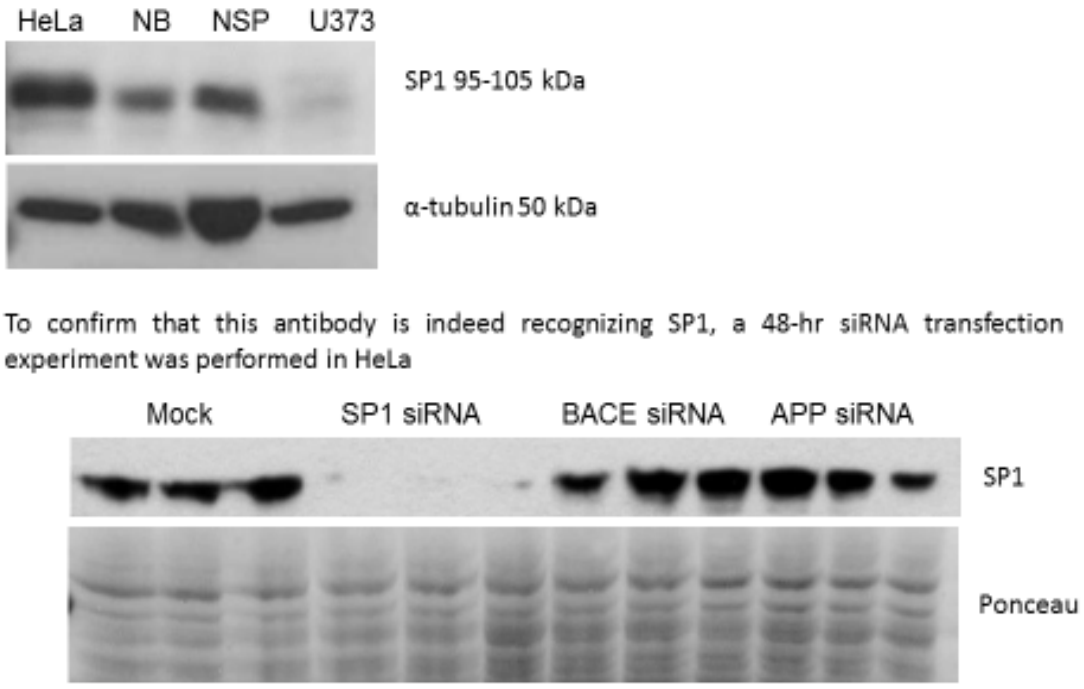


Figure 6. SP1 is differentially expressed in various CNS cell types. A) SP1 levels as detected by Western immunoblotting. Relative levels of SP1 in U373 are lower than that of HeLa, SK-N-SH (NB), or undifferentiated neurospheres (NSP). B) siRNA knockdown in various cell lines. Transfection of HeLa cells with SP1 siRNA confirms the authenticity of the SP1 band.

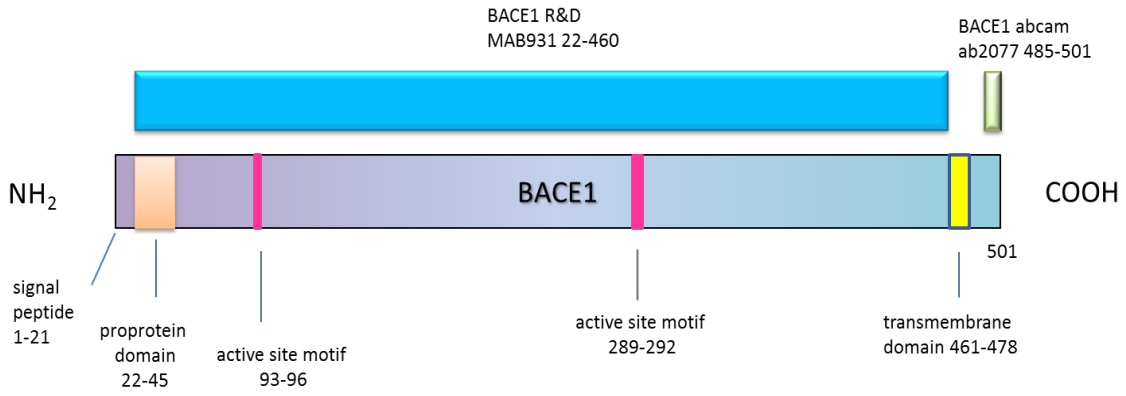


Figure 7. Schematic comparing two commercially available BACE1 antibodies. BACE1 from R&D and abcam recognize different epitopes of BACE1 protein. R&D detects between amino acids 22 – 460 and the abcam 485 – 501. *Source: Bayon, B.L., in preparation.*

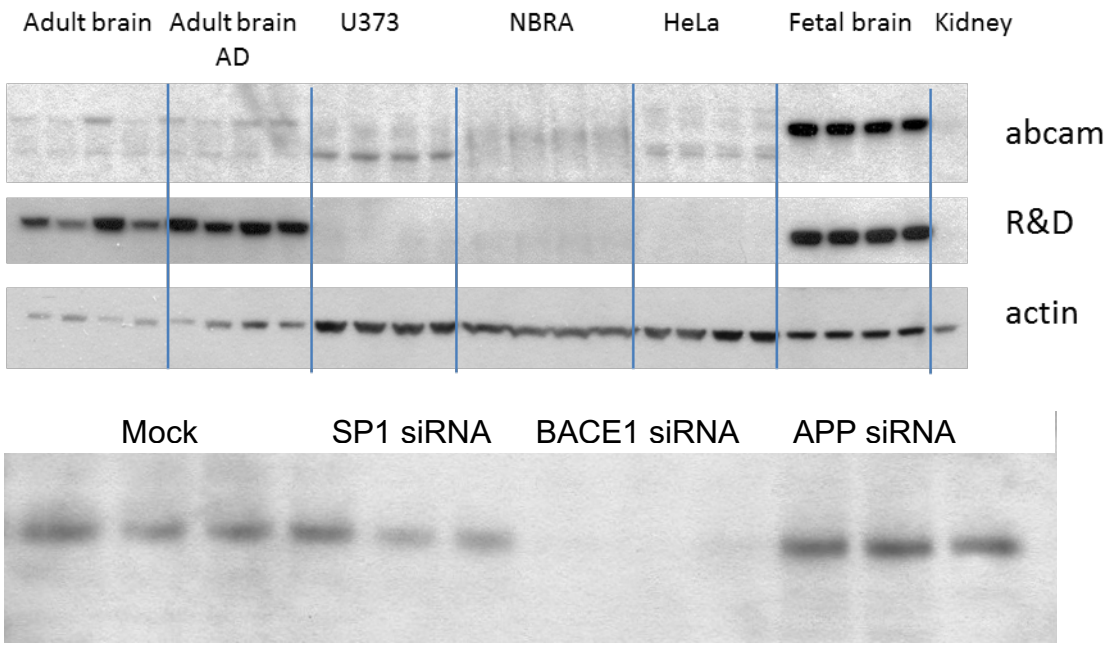


Figure 8. Comparison of two commercially available BACE1 antibodies and expression in various cell types. The abcam and R&D antibodies differentially detect BACE1 in adult brain, U373, NBRA, and HeLa. R&D antibody detects BACE1 at 60 kDa, abcam at 70 kDa. An siRNA experiment in U373 was used to confirm the authenticity of the BACE1 band seen with the BACE1 antibody from the Vassar lab in U373. A band was detected at nearly 75 kDa in all but the BACE1 siRNA transfected lysates. The abcam and Vassar lab antibody was used in subsequent experiments.

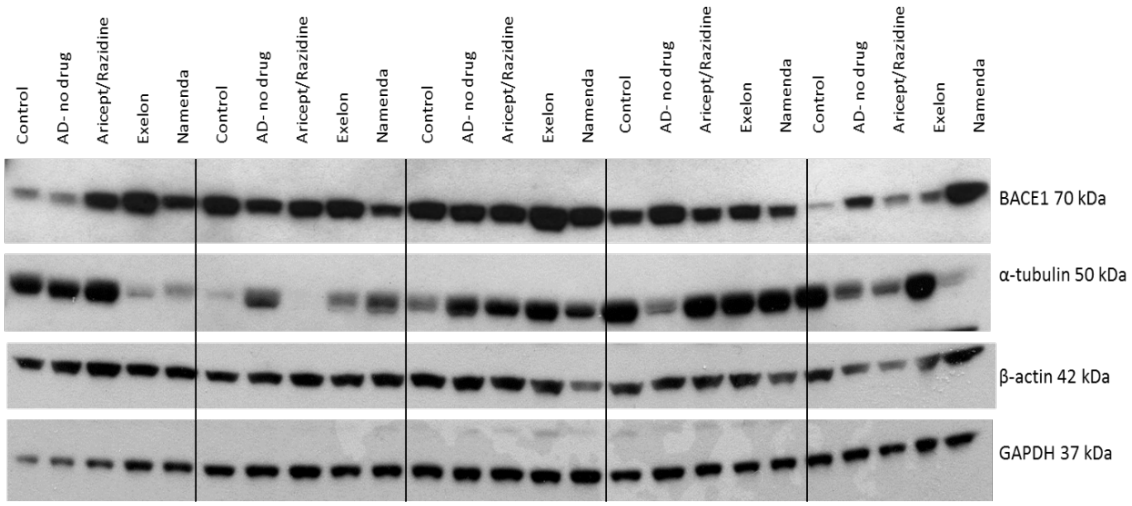


Figure 9. BACE1 expression in human brain samples. Control (n=5) and AD (n=15) human brain tissue samples examined by Western immunoblotting after SDS-PAGE. AD specimens further sub-grouped by exposure to AD therapeutics Aricept/Razidine, Exelon, Namenda, or no drug treatment. Alpha-tubulin, beta-actin, and GAPDH were used as loading controls. BACE1 (abcam) antibody detects protein at about 70 kDa in human adult brain. No significant changes were detected among controls versus AD subjects.

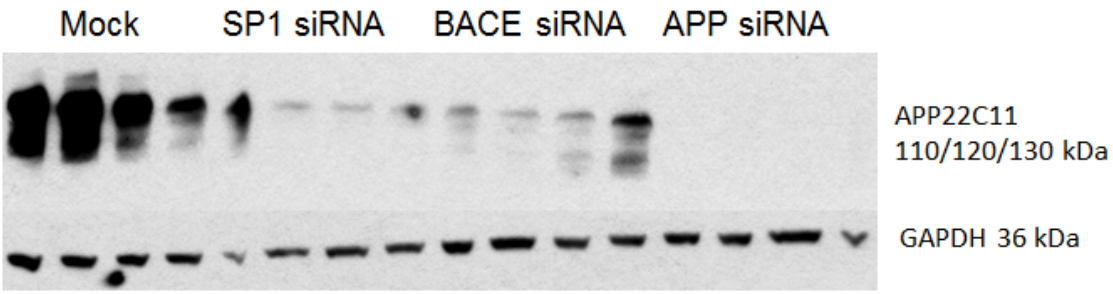


Figure 10. APP Expression in U373. The authenticity of the APP antibody was confirmed by siRNA transfection of U373 cells. siRNA transfected cells show complete knockdown of APP levels.

C. Immunocytochemistry (ICC)

After treatment, cells were washed with PBS in 24-well plates and fixed in 4% paraformaldehyde for 10 min. Wells were then washed twice with PBS to remove traces of paraformaldehyde. All PBS was removed, and then 0.25% Triton-X (Sigma) was added for 15 min to permeabilize the cell membranes. Wells were then washed 3 times with PBS to remove all traces of Triton-X. Nonspecific binding was then blocked using 10% horse serum in PBS for 30 min. Primary antibodies were applied at the antibody-specific dilutions as indicated in Table 1 in 1% horse serum overnight at 4°C. The following day, wells were washed 3 times for 5 minutes with PBS. Host-appropriate secondary antibodies were applied in 1% horse serum for 1 h and washed 3 times for 5 minutes with PBS. Secondary antibodies were conjugated to fluorescein isothyocyanate (FITC), cyanine-3 (Cy-3), or biotin. Biotinylated secondary antibodies were subsequently incubated with either Cy-3 or FITC conjugated streptavidin. Biotin and FITC conjugates were used at 1:300 dilutions, while Cy-3 secondary conjugates were used at a 1:1500 dilution. All secondary agents and streptavidin conjugates were from Jackson Immunoresearch.

D. Amyloid-beta 40 ELISA

ELISA kits were obtained from Invitrogen. The kits contained microwell plates pre-coated with capture antibodies, and HRP-labeled detection antibodies were provided in the same kit. Samples (50 µL) were added to the plate followed by detection antibody and allowed to incubate for 3 h at room temperature (RT). Wells were washed 4 times, then 100 µL of HRP anti-rabbit antibody was added

for an incubation time of 30 min at RT. Wells were washed 5 times, then 100 µL of stabilized chromogen was added for 30 min at RT. Lastly, 100 µL stop solution was added to wells and absorbance read at 450 nm.

E. Human Brain Samples

Two cohorts of adult human brain were used to measure relative protein expression levels. One cohort was obtained from the Nelson Lab and the other from the Reddy Lab. Samples were received and then processed in order to lyse proteins and kept in 1X Laemmli sample buffer for SDS-PAGE and Western immunoblotting.

Table 2. Reagents used in experiments

Reagent	Company	Catalog Number
10x FASTRun Tris SDS PAGE Running Buffer	Fisher	BP881-500
12-well Flate Bottom Culture Plate	Corning	3513
24-well Flat Bottom Culture Plate	Corning	3526
48-well Flat Bottom Culture Plate	Corning	3585
96-well Flat Bottom Culture Plate	Corning	3596
Albumin bovine fraction V	ICN Biomedicals	160069
all-trans-retinoic acid	Sigma	R2625
Ammonium persulfate	amresco	0486-256
B27	Gibco	17504-044
Blocker™ Casein in TBS	Thermo Scientific	37528
cellgro™ Eagle's Minimum Essential Medium (MEM)	Corning	10-010-CV
cellgro™ RPMI 1640 Medium	Corning	10-104-CV
CellTiter-Fluor™ Cell Viability Assay	Promega	G6080
CellTiter-Glo® Luminescent Cell Viability Assay	Promega	G7570
CL-XPosure Film	Thermo Scientific	34090
Collagen	Sigma	C7521
Criterion XT Bis-Tris Gel	BioRad	345-0113
Cytotoxicity Detection Kit (LDH)	Sigma-Aldrich	11644793001
Dimethyl sulfoxide (DMSO)	Santa Cruz	sc-358801
DNMT Activity/Inhibition Assay	Active Motif	55006
Donor Horse Serum	Cellgro	35-030-CV
Fetal Bovine Serum	Cellgro	35-015-CV
FGF-Basic (AA 1-155) Recombinant Human Protein	Life Technologies	PHG0264
Gibco™ Opti-MEM™ I Reduced Serum Media	Life Technologies	31985070
Glutamax	Life Technologies	35050061
HDAC (Colorimetric) Assay Kit	Active Motif	56210
Hoescht stain	Invitrogen	H1399
Human Aβ40 ELISA Kit	Invitrogen	KHB3481
Human BDNF Recombinant Protein	Cell Signaling	3897
iBlot® Transfer Stack, PVDF, regular size	Invitrogen	IB401001
Lipofectamine® RNAiMAX Transfection Reagent	Invitrogen	13778030
Luc Photo TF Activation Reporter Assay	Signosis	BA-1001
Luciferace Assay System	Promega	E1500

Mithramycin A	Santa Cruz	sc-200909
M-PER™ Mammalian Protein Extraction Reagent	Thermo Scientific	78501
MTM-SDK	Ratan Lab	
MTM-SK	Ratan Lab	
Neurobasal Medium	Life Technologies	12348-017
Neurocult NS-A Basal Media	StemCell Technologies	5750
Neuronal Growth Factor (NGF)	Life Technologies	11050HNAC50
Normocin	invivogen	ant-nr-1
One-Step TF Activation Reporter Array I	Signosis	BA-0001
Paraformaldehyde	Sigma-Aldrich	P6148
Penicillin-Streptomycin Solution	Cellgro	30-001-CL
Phosphate-Buffered Saline (10X)	Cellgro	20-031-CV
Pierce™ BCA Protein Assay Kit	Thermo Scientific	23227
Pierce™ ECL Western Blotting Substrate	Thermo Scientific	32106
Pierce™ RIPA Lysis & Extraction Buffer	Thermo Scientific	89900
Poly-D-lysine	Sigma	P6407-5MG
Ponceau S Stain	Fisher	BP103-10
Precision Plus Protein™ Dual Color Standards	BioRad	161-0374
Protease Inhibitor Complete Mini	Roche	11836153001
Qproteome Nuclear Protein Kit	QIAGEN	37582
Restore Stripping Buffer	Thermo Scientific	46430
Roscovitine	Sigma	R7772
Silencer Select siRNA (APP)	ambion	s13318
Silencer Select siRNA (BACE1)	ambion	s24218
Silencer Select siRNA (SP1)	ambion	s13318
SuperSignal™ West Femto Maximum Sensitivity Substrate	Thermo Scientific	34095
SuperSignal™ West Pico Chemiluminescent Substrate	Thermo Scientific	34077
TF Activation Profiling Plate Array I	Signosis	FA-1001
Tolfenamic Acid	Sigma	T0535-5G
Tris Buffered Saline (TBS)	Cellgro	46-012-CM
Triton X-100	Sigma	X100-100ml
Trypsin-EDTA 1X	Cellgro	25-053-CI
TWEEN 20	Calbiochem	655205
XT-MOPS	BioRad	161-0788

III. Experimental Methods

A. Statistical analysis

All experiments were performed at least three times unless otherwise indicated. Treatments and assay loading was done with sample randomization in plates and gels to avoid bias. Experiments described were repeated to confirm reproducibility. Data are expressed as mean ± s.e.m. Statistical analysis was performed with GraphPad Prism (version 5.04, GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was assumed if $P \leq 0.05$. Statistically significant differences were determined by ANOVA followed by post-hoc Dunnett's correction for multiple comparisons.

B. Primary tissue culture

Cell culture models are useful, especially when the aim is to observe the effects of pharmacological treatments on particular cell or tissue types. Cell culture models allow evaluation of secreted molecules into the culture medium without the factor of clearance that is present with *in vivo* models. For instance, it may be difficult to determine if Aβ peptide measurements in an animal are due to a change in production or a change in clearance after an experimental manipulation [141]. Morphological measurements are also an advantage to tissue culture models, especially with the live cell imaging technology of the Incucyte Zoom (See Methods). Cells form a single layer in culture, allowing morphology to be observed in a single plane.

i. Preparation of tissue culture plates

Tissue culture plates were coated with poly D-lysine (PDL). Powdered PDL was reconstituted in sterile water at 100 µg/mL, sterile filtered, and added to the center of each well of the tissue culture plates. PDL was left on the plates overnight in the cell culture hood under UV light. The following day, PDL was removed and wells were rinsed with sterile water. Plates were allowed to dry for 30 min before plating cells.

ii. Tissue culture maintenance

Cultures were maintained in a sterile tissue culture incubator at 5% CO_2 atmosphere at 37°C. Cell culture media was changed as needed (typically every 2-3 days). At the conclusion of experiments, media samples were collected, cells were rinsed in 1X Dulbecco's phosphate buffered saline (PBS; Invitrogen) to remove traces of media protein. Cells were then lysed in RIPA buffer.

iii. Isolation of neurospheres

Neurospheres have been generated from various regions of the fetal CNS of various individuals. This methods section describes the broad, overall methodology used to generate neurospheres from human primary mixed brain from fetuses at a gestational age between 90-110 days (Figure 12). Preparation of human primary mixed brain cultures has been previously described [139]. The protocol used in our laboratory was approved by the Institutional Biosafety Committee (IRB) of Indiana University School of Medicine in Indianapolis, IN and conform to NIH guidelines. Human fetal brain tissue were obtained from legally elective aborted fetus from the Laboratory of Developmental Biology in Seattle,

WA. Brain tissues were shipped from the abortion clinic to our research laboratory in cold shipping media.

Briefly, 100 mm tissue culture plate containing 5 mL of fresh shipping media was placed on ice. Brain tissue was transferred to the fresh media using a wide-bore siliconized Pasteur pipette. Visible blood vessels were removed and cleaned tissue was sliced with a scalpel to obtain 0.5 mm sections of tissue. These brain tissue sections were transferred into a 50 mL PET tube containing 10 mL trypsin-EDTA. This tube was placed in the water bath at 37°C and shaken at 150 RPM for 10 minutes to enzymatically digest the tissue. This cell mass was then transferred by wide-bore glass Pasteur pipette into a 15 mL PET tube containing 5 mL media. The mass was then triturated 10 times to obtain a homogenous suspension of cells. Tubes were centrifuged at 400 g at room temperature for 5 min. Supernatant was discarded and fresh shipping media was added into the tubes. This trituration and centrifugation step was repeated, supernatant was removed, and 3 mL fresh shipping media was added. Homogenate was mixed by slow pipetting with a P1000 micropipette. The resulting cell suspension (20 µL) was mixed with 180 µL of sterile PBS. A sample was removed for counting by trypan blue exclusion. Cells for HFN were plated in PDL-coated plates at this step in defined culture medium (Neurobasal with supplements and antibiotics). For neurosphere culture, however, this suspension was placed into flasks containing NeuroCult Proliferation Media supplemented with bFGF (10 ng/mL). Half media changes were made every 48 h until cells were ready for differentiation.

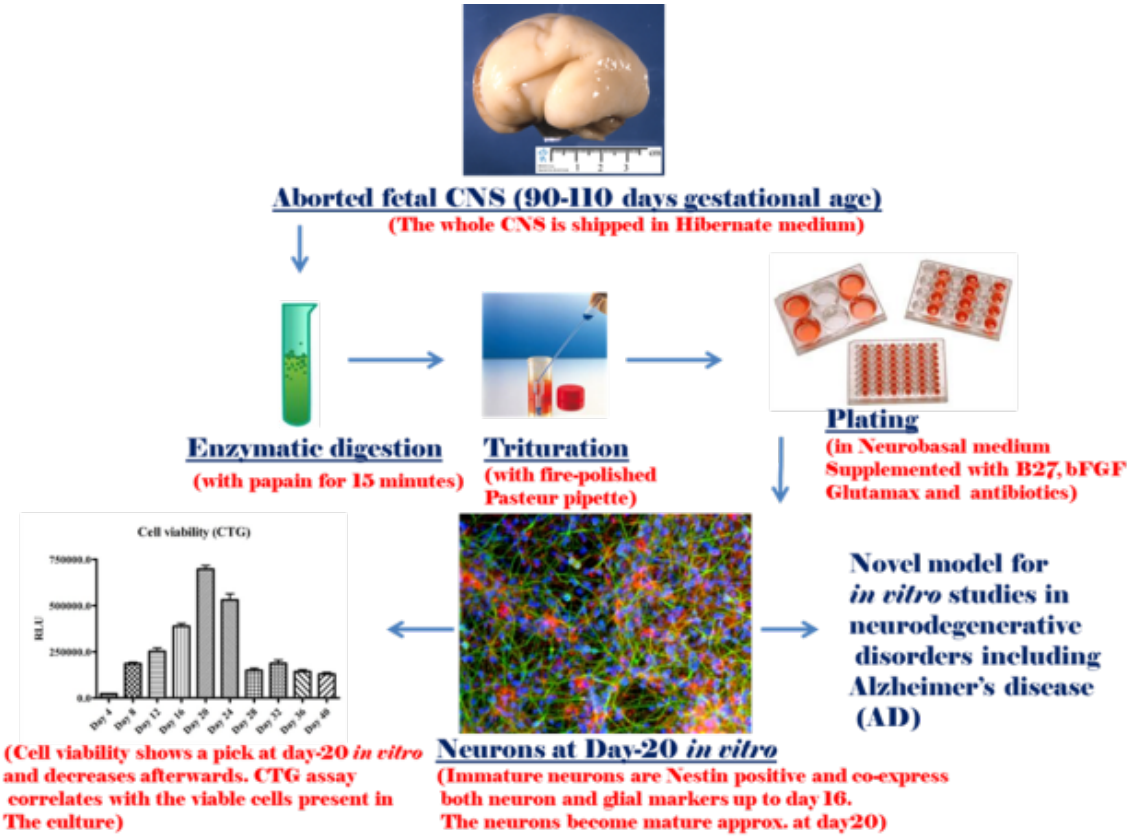


Figure 11. Processing of human CNS. Whole CNS was enzymatically digested, triturated, and either plated to yield human fetal neuron (HFN) culture, or placed into proliferation media to isolate neurospheres. Ray, B., et al. Molecular Brain 2014 (7) 63.

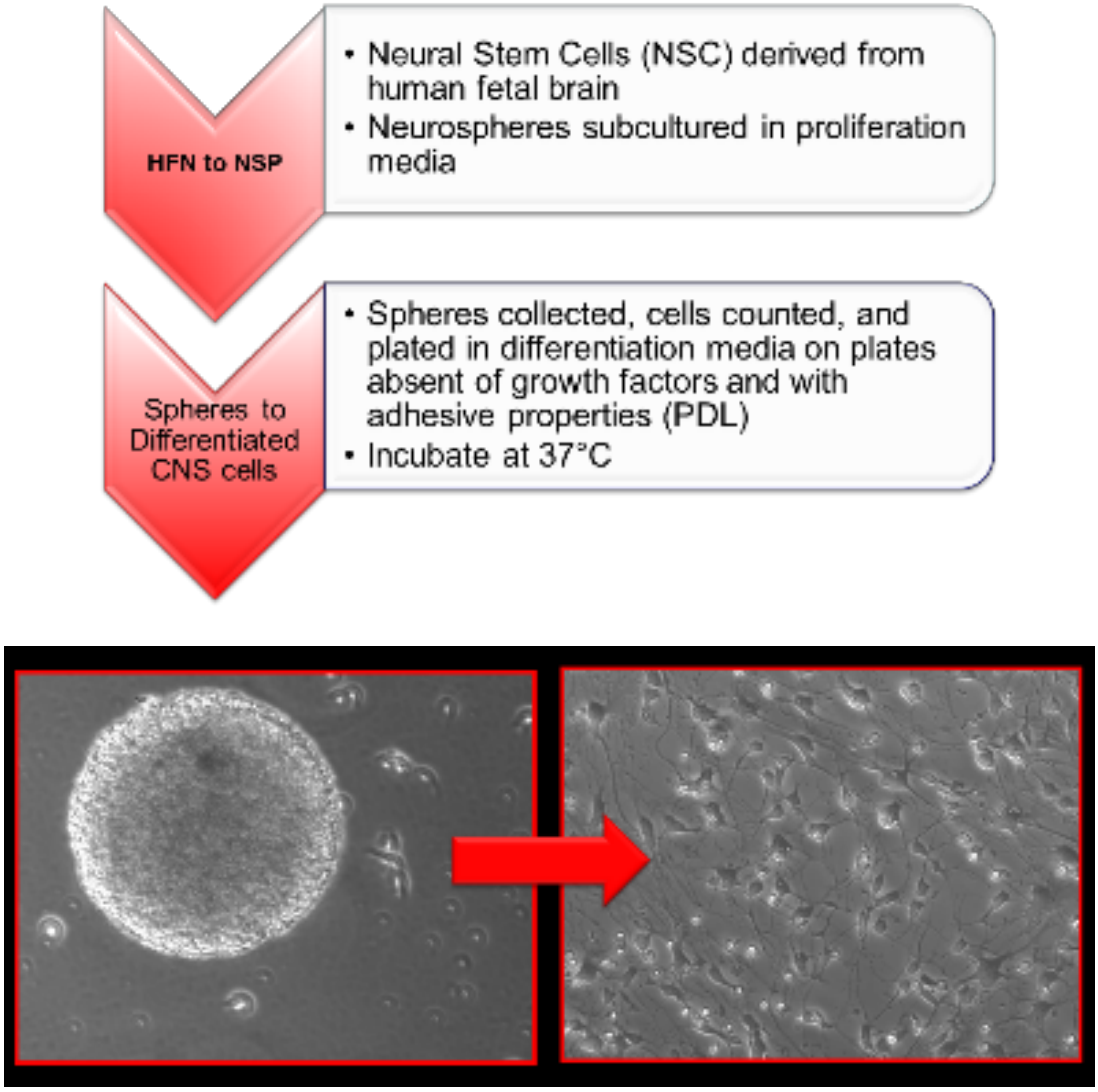


Figure 12. Neurospheres derived from human CNS. Free floating neurospheres were cultured in proliferation media. Spheres were plated in differentiation media on plates treated with poly-D-lysine and allowed to mature for 10 days before being treated with drug or transfected with siRNA.

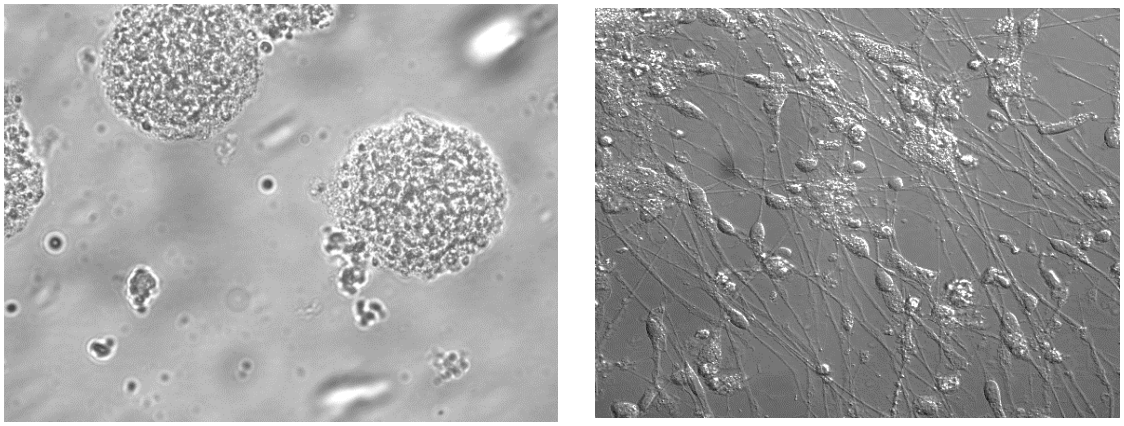


Figure 13. Human neurospheres in proliferation media vs. differentiation media. Free floating neurospheres form adherent, single layer cultures while differentiating.

iv. Characterization of neurosphere culture

We have previously cultured and characterized primary human neuron culture [142]. Neurospheres were isolated from fetal human brain tissue (Figure 13). Cells were cultured and maintained in Neurocult NS-A Basal Media (Stem Cell Technologies #05750) supplemented with Neurocult proliferation supplement in flasks for up to 5 days (Figure 14).

To optimize media conditions for subculturing neurospheres, undifferentiated neurospheres in proliferation media with and without growth factors were harvested at several time points; day in vitro (DIV) 7, 14, 21, and 28. Cells were cultured in flasks in either proliferation media alone or proliferation media supplemented with EGF and bFGF for 14 days. At DIV15, BDNF was added to both flasks (Figure 15). Cells were collected and lysed for use in cell viability analysis using the Cell Titer Glo (CTG) assay (see Methods). Neurospheres cultured in media containing bFGF, EGF, and BDNF show a significant increase in cell viability up to DIV21. By DIV28, cell viability was significantly decreased, signaling that cells should not be cultured past DIV21 in future experiments and should always be in media supplemented by bFGF and EGF. Western blot analysis was performed to demonstrate protein levels of nestin (neural progenitor marker), NSE (neuronal marker), and SNAP25 (synaptic marker) in these neurospheres over time (Figure 16). When supplemented with bFGF and EGF, the neuroprogenitor population was maintained up to 14 days and up to 21 days with the addition of BDNF. However, this decreases at DIV28. The mature cell protein levels were

consistent across various time points and regardless of absence or presence of growth factor supplements. Synaptic protein expression was significantly decreased at DIV21 with the addition of BDNF in cultures supplemented with bFGF and EGF. In future experiments, cells were cultured closer to DIV14 and in media supplemented by bFGF and EGF.

Primary neurospheres were mechanically dissociated to a single-cell suspension and plated in media supplemented with Neurocult differentiation supplement, media plus 10 ng/mL bFGF, media plus 10 ng/mL BDNF, or media plus 10 ng/mL bFGF and 10 ng/mL BDNF. Cells were plated in PDL-coated 24-well plates, and cultured at 37°C for 21 days. Cell morphology was monitored by phase contrast microscopy throughout. We collected cell lysates at two time points; DIV 11 and 21 for use in cell viability analysis. CTG revealed a significant decrease in cell viability in all media besides Neurocult media containing Neurocult Differentiation Supplement (Figure 17). This was true at both DIV11 and DIV21. CTG also showed that cells cultured in Neurocult media plus Neurocult Differentiation Supplement experience no significant change in cell viability after 10 days (Figure 17). On the other hand, cells in media supplemented with bFGF, BDNF, or a combination of both growth factors experienced significant decreases in cell viability between DIV11 and DIV2. These data revealed that although proprietary, the differentiation supplement provided commercially by StemCell Technologies maintains differentiated NSCs with the highest cell viability compared to other growth factors. This recipe was used for future experiments.

Cells were cultured and plated in Neurocult media plus Neurocult Differentiation Supplement in a 24-well format. Cell lysate was collected from cells at each time point (n=4; DIV5, 10, 15, 20 plate 1; DIV30, 35, 40, 45 plate 2). CTG reveals a time dependent decrease in cell viability from DIV5 to DIV20 (Figure 18). However, cell viability increased at DIV40 before decreasing at DIV45. These data showed that dNSPc can be maintained for several weeks with relatively high cell viability as compared to that of cells plated for only a few days.

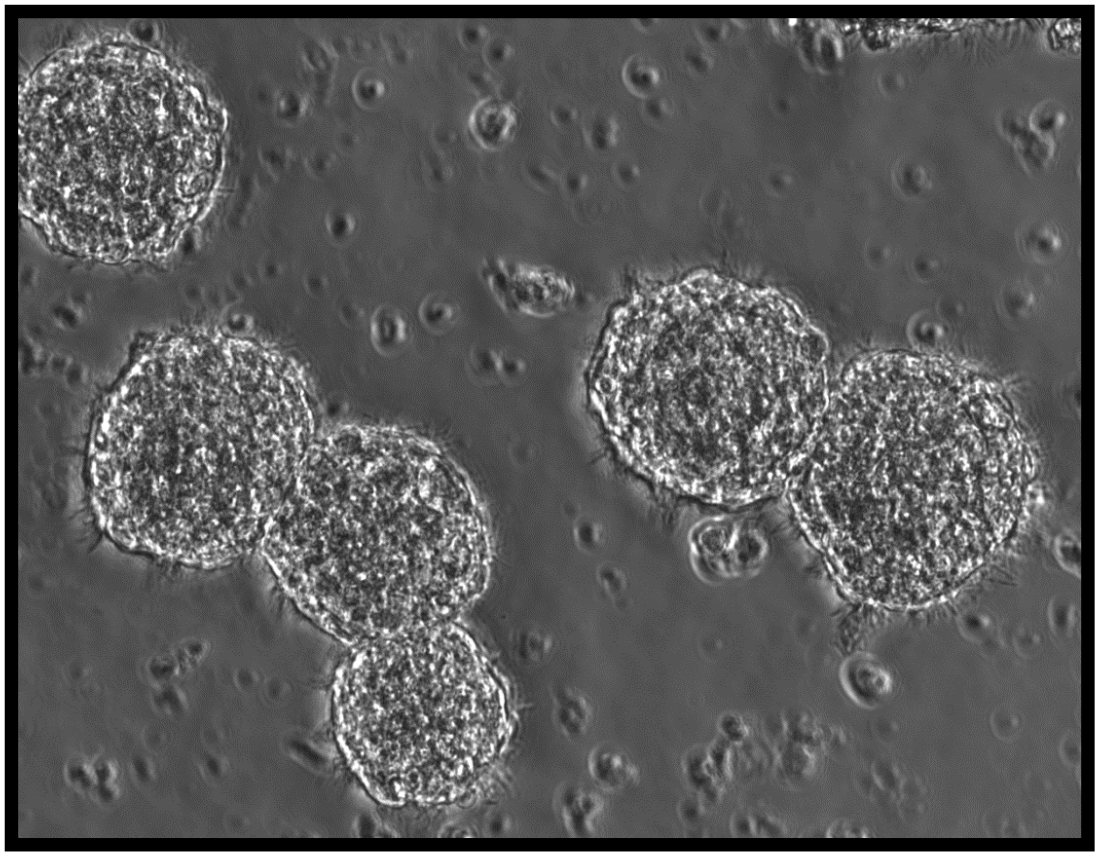


Figure 14. Undifferentiated neurospheres in proliferation media. Free floating neurospheres in culture are clusters of neuronal stem cells. These neurospheres are comprised of varying percentages of neural stem cells. Neurospheres such as these were dissociated and distributed evenly into plates in order to be differentiated for use in drug treatments.

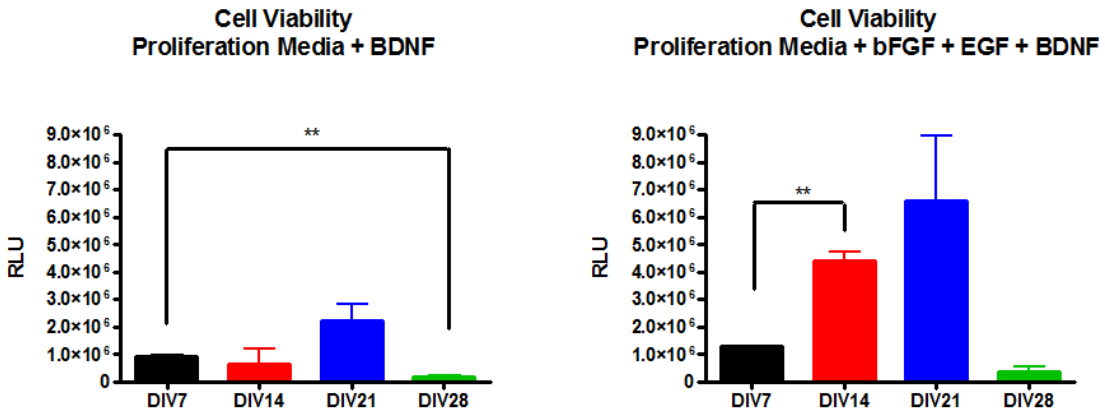


Figure 15. Undifferentiated NSPc in proliferation media supplemented with growth factors. Isolated undifferentiated neurospheres plated in proliferation media OR proliferation media plus bFGF and EGF for 14 days; BDNF added at DIV15. Near 5 fold increase in cell viability of NSP from DIV7 to DIV14 when media was supplemented with bFGF and EGF; while no change was seen at this time interval in media alone

(a)

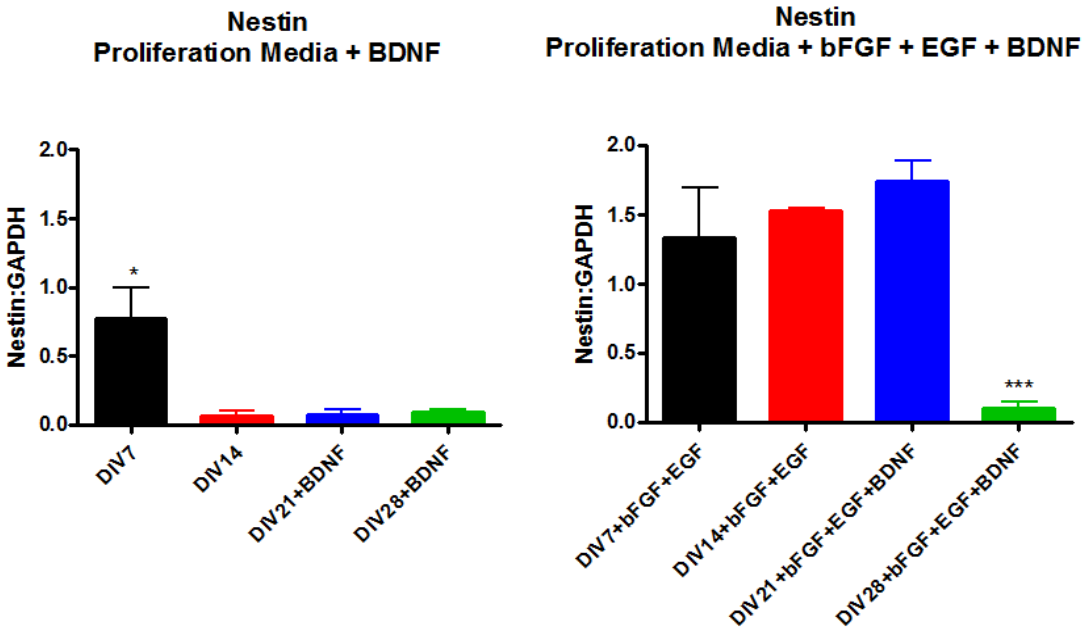


Figure 16a. Optimizing media conditions by protein levels detected by Western immunoblotting. When supplemented with bFGF and EGF, the neuroprogenitor population was maintained up to 14 days and up to 21 days with the addition of BDNF. However, this population decreased at DIV28.

(b)

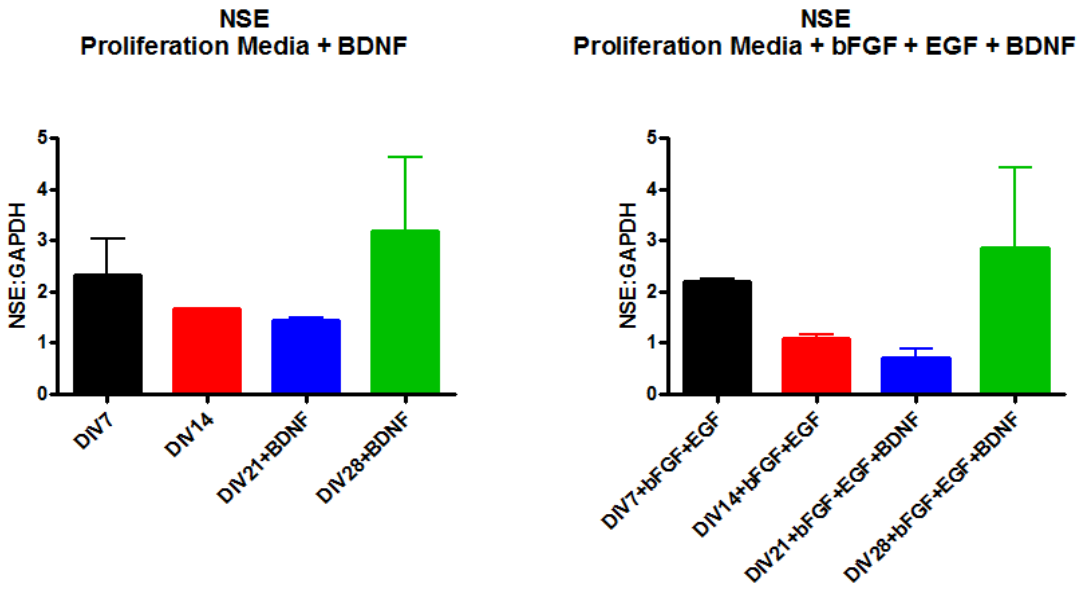
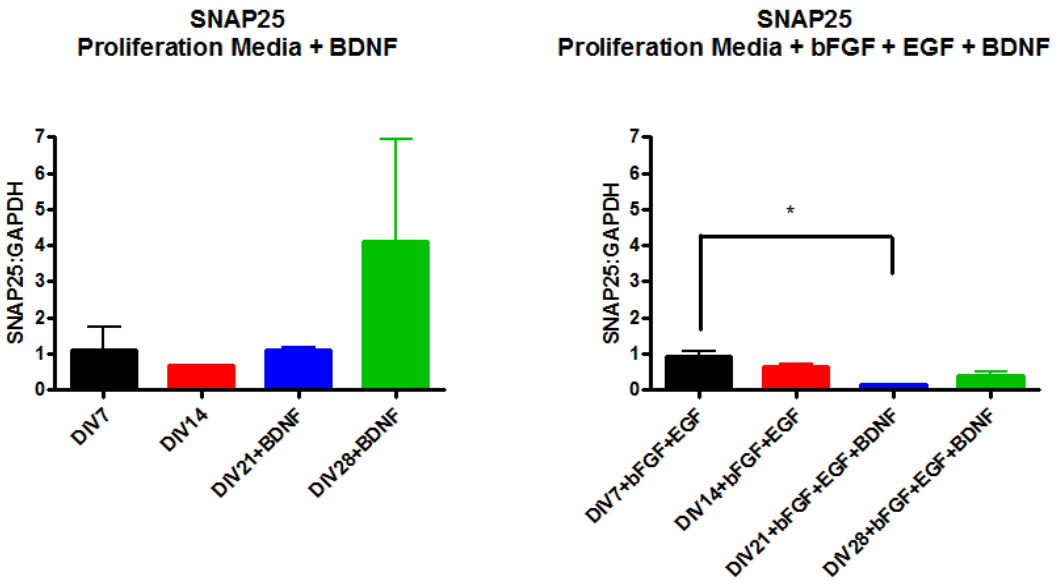
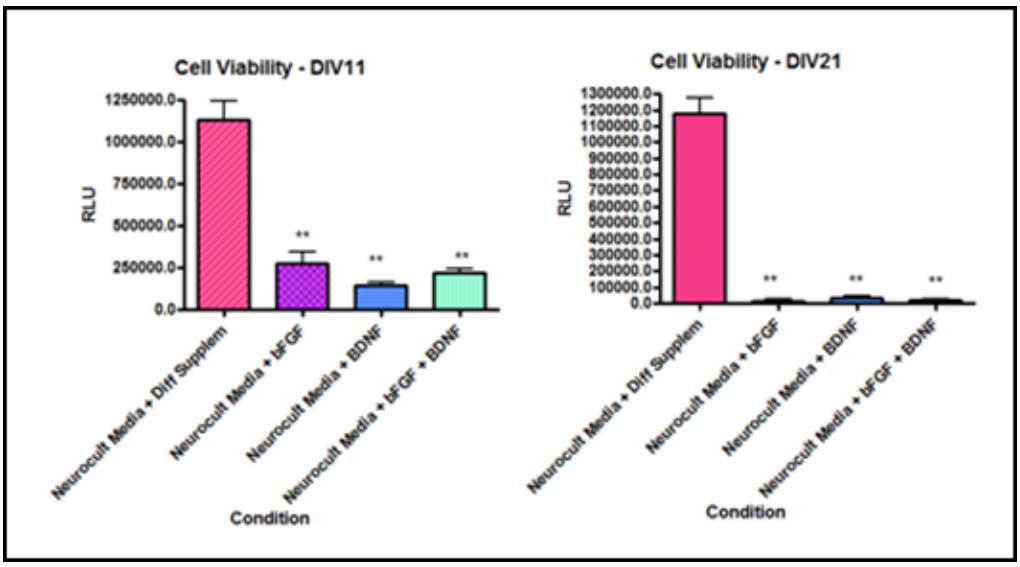


Figure 16b. Optimizing media conditions by protein levels detected by Western immunoblotting. The mature cell protein levels were consistent across various time points and regardless of absence or presence of growth factor supplements.



(c)

Figure 16c. Optimizing media conditions by protein levels detected by Western immunoblotting. Synaptic protein expression was significantly decreased at DIV21 with the addition of BDNF in cultures supplemented with bFGF and EGF.



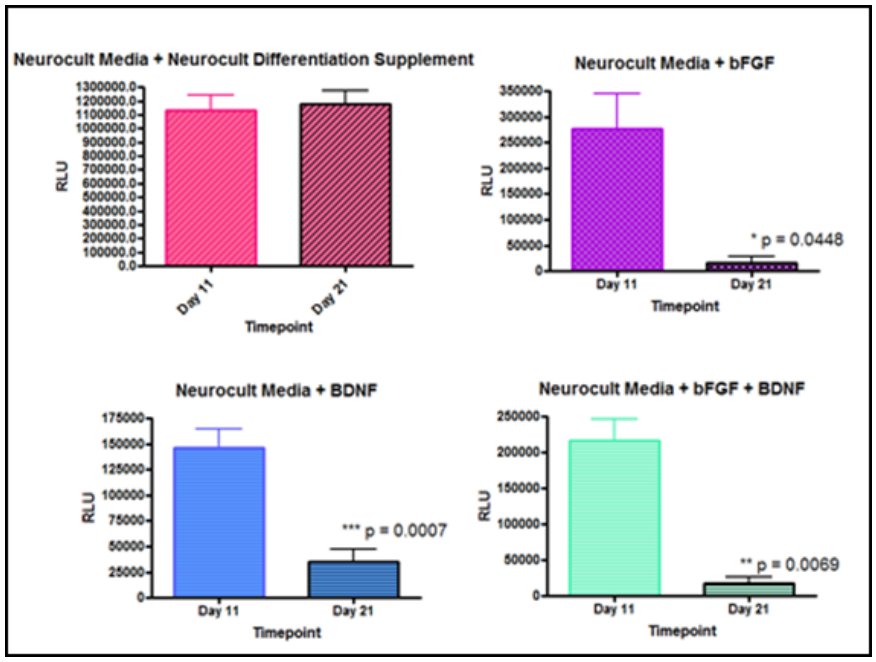


Figure 17. Optimization of media conditions for differentiated neurospheres. Media with commercially available differentiation supplement yielded the highest cell viability among tested conditions.

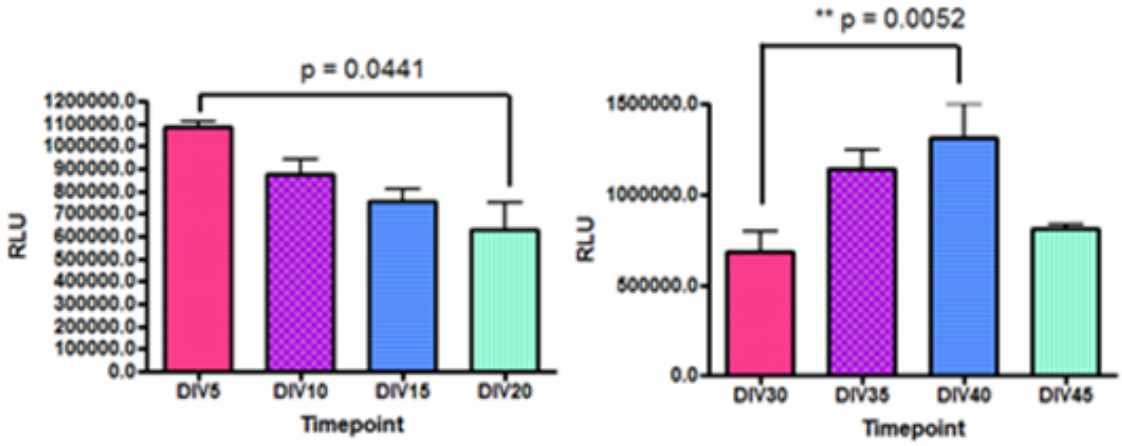


Figure 18. Extended culture times reveal an increase in cell viability in differentiated neurosphere culture. dNSPc were harvested at 8 time points. Cell viability significantly decreased at DIV20, yet increased at DIV40 before decreasing at DIV45. These data showed that dNSPc can be maintained for several weeks with relatively high cell viability as compared to that of cells plated for only a few days.

v. Protein marker characterization by Western immunoblot

Lysates of cells maintained in neurosphere culture before differentiation were subjected to Western blotting to determine relative expression levels of relevant proteins.

a. Undifferentiated neurosphere culture

Free floating neurospheres were cultured in proliferation media containing bFGF or bFGF plus BDNF for 14 days. At DIV7 and 14, cells were collected, harvested, and lysed for SDS-PAGE and Western blot analysis. Significant basal levels of the astrocytic marker GFAP were consistent from DIV 7 and 14 (blot not shown). Neuronal precursor and immature markers nestin and doublecortin were used to assess the neuroprogenitor protein expression in these cells (Figure 19). Post-synaptic marker PSD95 was detected at each time point as well as neuronal marker NSE (Figure 20). Amyloid pathway proteins APP and BACE1 are detected in undifferentiated NSP (Figure 21).

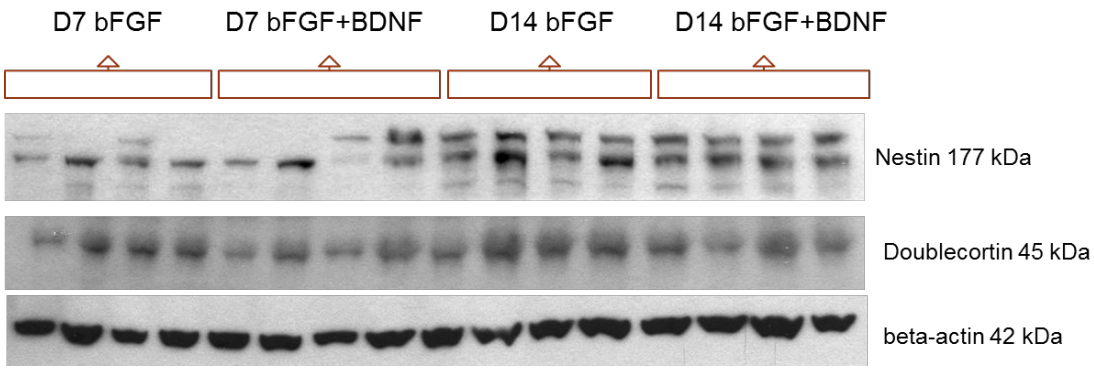


Figure 19. CNS stem cell and neuronal precursor/immature neuronal marker expression of undNSPc. Western immunoblot of lysates from undifferentiated neurosphere culture. Cells were allowed to proliferate in media supplemented with bFGF alone, or with bFGF and BDNF combined for up to 14 days. Lysates were collected at two time points, DIV7 and DIV14. Nestin, an intermediate filament expressed in developing neurons, was detected at both time points. Doublecortin is a neuronal migration protein expressed by immature neurons and neuronal precursors. Consistent levels of doublecortin were detected at both time points as well.

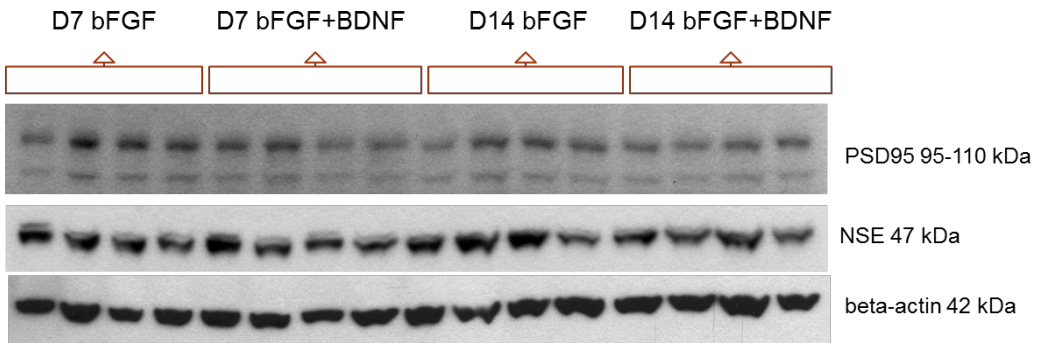


Figure 20. Post-synaptic and mature neuronal marker expression of undNSPc. Western immunoblot of lysates from undifferentiated neurosphere culture. Cells were allowed to proliferate in media supplemented with bFGF alone, or with bFGF and BDNF combined for up to 14 days. Lysates were collected at two time points, DIV7 and DIV14. PSD95 is a scaffolding protein located in the post-synapse of neurons. NSE is an enzyme found in mature neurons. Both postsynaptic and mature neuronal markers were detected in undNSPc at DIV7 and DIV14.

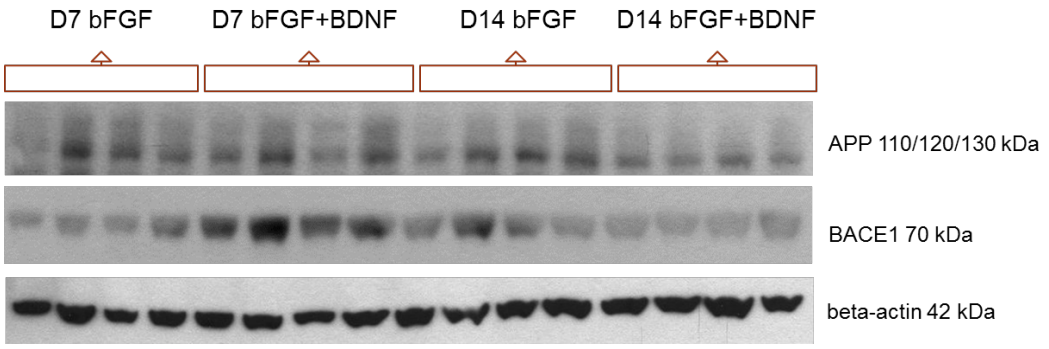


Figure 21. High basal levels of APP and BACE1 expressed in undNSPc. Western immunoblot of lysates from undifferentiated neurosphere culture. Cells were allowed to proliferate in media supplemented with bFGF alone, or with bFGF and BDNF combined for up to 14 days. Lysates were collected at two time points, DIV7 and DIV14. Expression of BACE1 increased with the addition of BDNF to proliferation media up to DIV7 and decreased at DIV14. APP was detected at both time points as well.

b. Differentiated neurosphere culture

Free floating neurospheres were differentiated as previously mentioned for 14 days, lysed, and proteins were separated by SDS-PAGE. Astrocytic and synaptic proteins were also detected in differentiated cultures along with neuronal markers (Figure 22). Western immunoblotting was used to detect amyloid-pathway proteins APP and BACE1 (Figure 23).

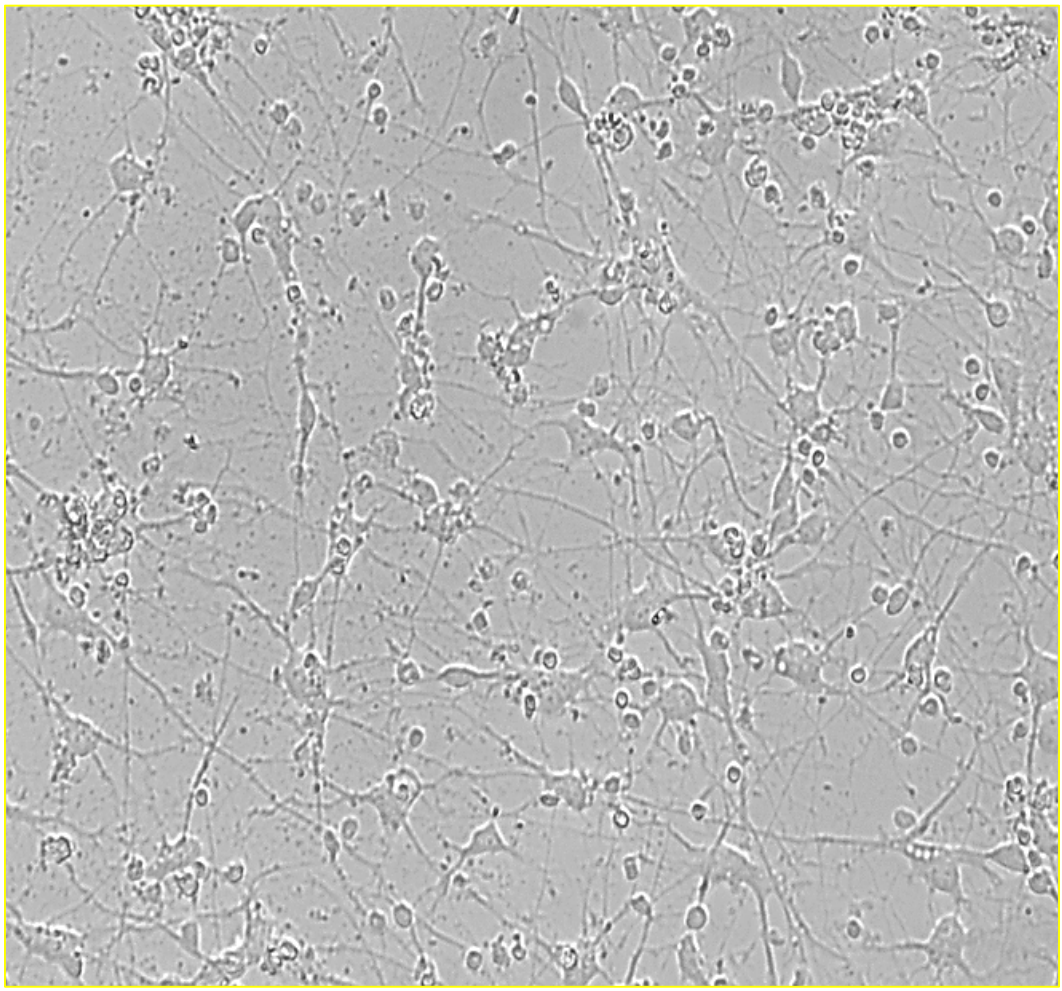


Figure 22. DIV 10 differentiated neurosphere culture. Free floating neurospheres in proliferation media were dissociated and counted. Cells were then transferred to differentiation media and plated at a density of 150,000 cells/well for 10 days.

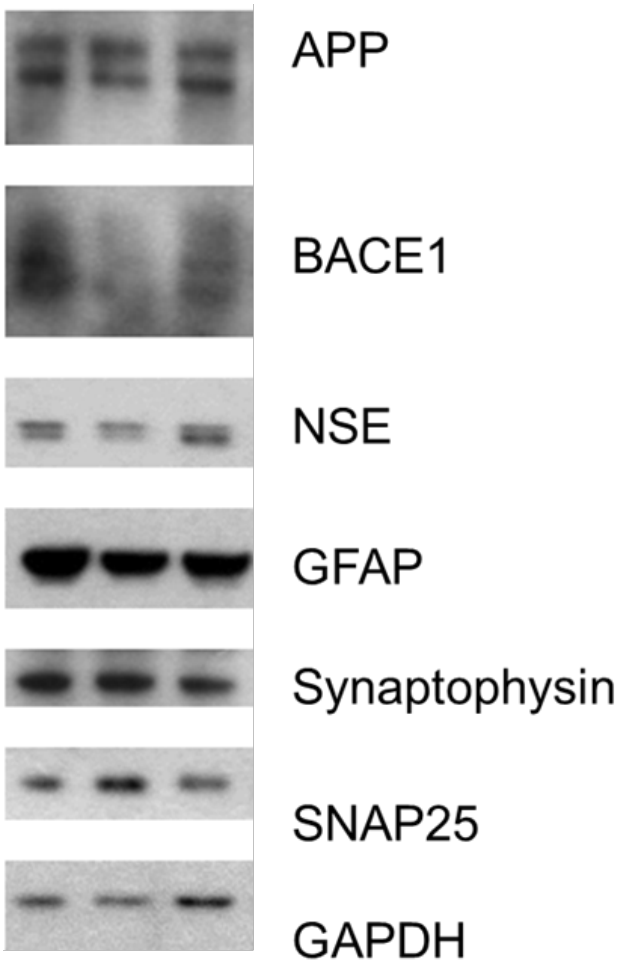


Figure 23. Western blot analysis shows human differentiated neurospheres express synaptic, neuronal, and astrocytic protein at Day 14. Proteins of the amyloid pathway, APP and BACE1 were detected in dNSPc. There was synaptic protein expression as detected by synaptophysin and SNAP25. Neuronal marker NSE levels were also detected in dNSPc. GAPDH was used as a loading control.

vi. Location of proteins by immunocytochemistry

Cells were fixed and stained as described above for ICC. Three time points of DIV7, 14, 21 were chosen (Figure 24). GFAP, pan-neuronal and nuclear (Hoechst) antibodies were used to visualize their localization and relative abundance in dNSPc culture (Figure 25). The astrocytic (GFAP+) population was maintained in differentiated neurospheres through Day 21 while the pan-neuronal (somatic, nuclear, dendritic, axonal protein marker cocktail) population decreased by Day 14.

vii. Assessment of cell phenotype by flow cytometry

Neuronal, glial, and other relevant antibody markers were used to assess the different cellular populations in the neurosphere cell cultures. Cells were fixed using 4% paraformaldehyde and permeabilized with 0.25% Triton X-100. Cells were then blocked with 10% horse serum and incubated overnight with the primary antibody of choice. Appropriate corresponding secondary antibodies and fluorophores were used.

Day 7, 14, 30 were time points used to assess differentiated cultures. The immature cell population as detected by nestin labeling of differentiated neurosphere culture decreased from nearly 60% at day 7 to almost 40% by day 30 as measured by flow cytometry (Figure 26). Mature cell population (NSE antibody labeling) of differentiated neurosphere culture appeared consistent (about 40%) at each time point as measured by flow cytometry (Figure 27).

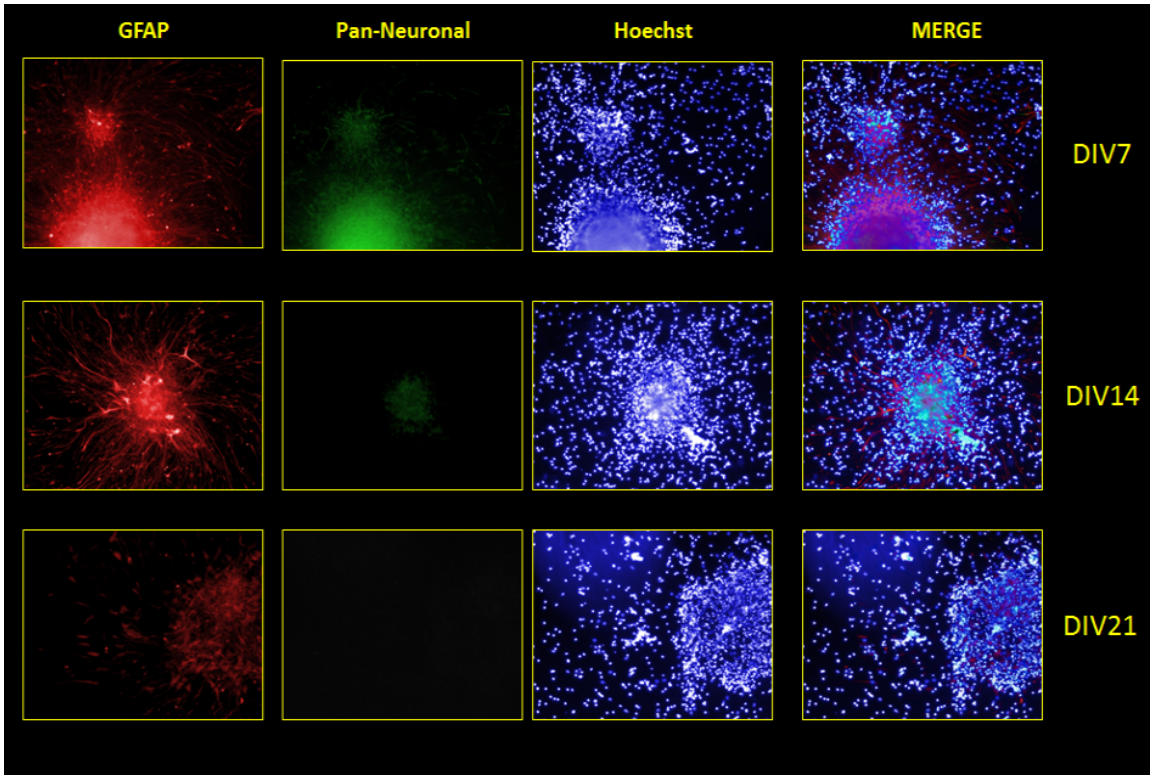


Figure 24. Immunocytochemistry of differentiated neurospheres at DIV7, 14, and 21. ICC shows astrocytic (GFAP) population is maintained in differentiated neurospheres through Day 21; Pan-Neuronal (somatic, nuclear, dendritic, axonal protein marker cocktail) decreases by Day 14.

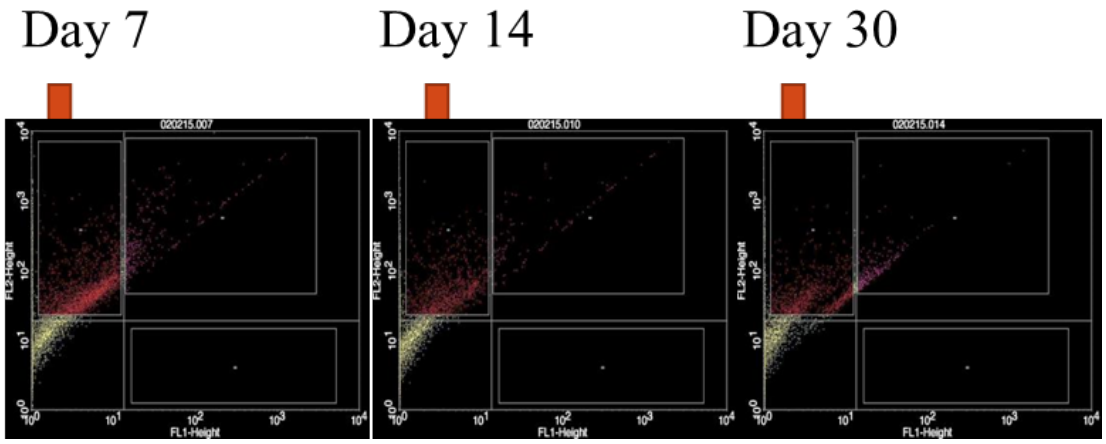


Figure 25. Immature cell population of differentiated neurosphere culture decreases from nearly 60% at day 7 to almost 40% by day 30 as measured by flow cytometry (Nestin labeling)

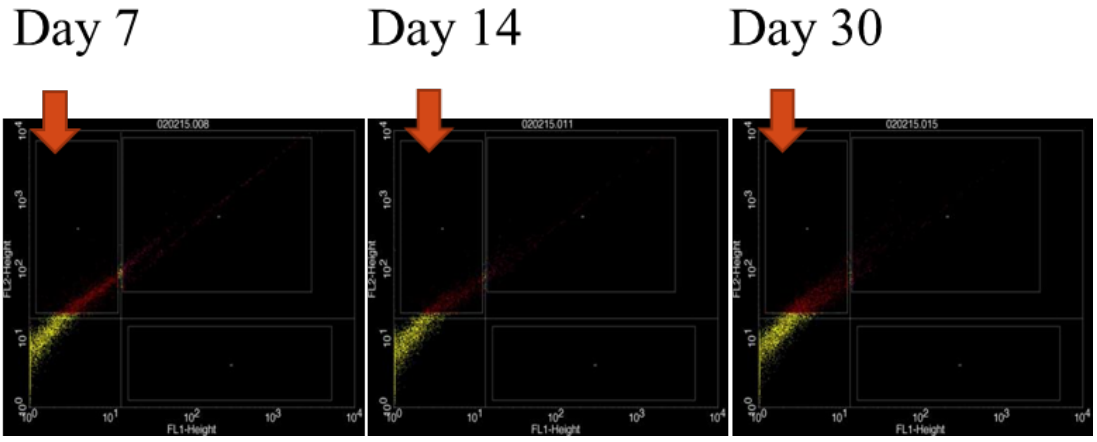


Figure 26. Mature cell population of differentiated neurosphere culture appears consistent (about 40%) at each time point as measured by flow cytometry (NSE labeling)

The population of undNSPc was analyzed by flow cytometry at DIV7 and 14. Addition of BDNF decreases the neuroprogenitor (double labeled GFAP and pan-neuronal) cell population of undifferentiated NSPc by nearly 15% represented by the upper right quadrant (Figure 28a). While maximum maturation level of the cell population (Pan-Neuronal labeled) of undNSPc by Day14 (Figure 28b).

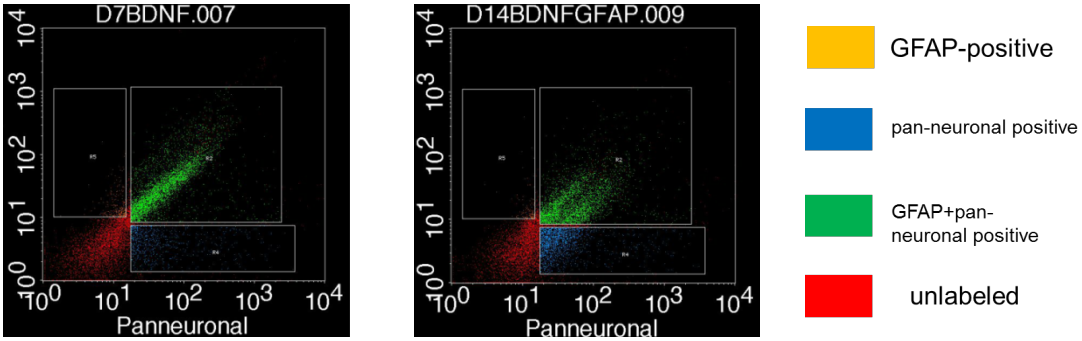


Figure 27a. Population of undifferentiated NSP as detected by flow cytometry. Upper left quadrant represents the GFAP positive cell population, of which there are very few represented at both DIV7 and 14 time points. Addition of BDNF decreases the neuroprogenitor (double labeled GFAP and pan-neuronal) cell population of undifferentiated NSPc by nearly 15% represented by the upper right quadrant. The lower right quadrant shows a pan-neuronal population that increases from DIV7 to 14.

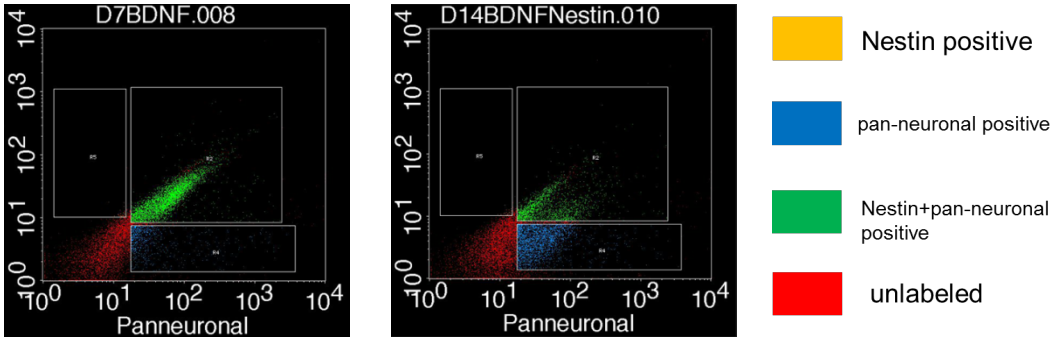


Figure 27b. Population of undifferentiated NSP as detected by flow cytometry. Flow cytometry shows maximum maturation level of the cell population of undifferentiated NSPc is achieved in proliferation media plus or minus BDNF by Day14 (Pan-Neuronal labeled).

C. Viability and toxicity assays

i. Cell Titer-Glo

To measure cell viability, the luciferase based Cell Titer-Glo kit (CTG; Promega, Madison, WI) was used to compare relative ATP concentration from cells in our experiments. The CTG assay was used to detect differences in cell density between wells. After cells were collected from the culture plate and lysed, 5 µL of lysate, 25 µL PBS, and 30 µL of CTG assay solution were added to a white bottom plate. The plate was placed on an orbital shaker for 10 minutes. Luminescence was measured with a Glomax luminometer (Promega) and reported at relative light units (RLUs).

ii. Lactose Dehydrogenase Assay

The lactose dehydrogenase (LDH) assay was used to quantify levels of LDH released by cells into the medium. LDH is a cytosolic enzyme that is released into the medium in proportion to the number of damaged and dead cells in culture. The commercially available LDH Cytotoxicity Kit (Roche) was used according to manufacturer protocol. To quantify the amount of LDH released by the cells, samples were compared to a standard curve of known LDH concentration (Figure 29). To determine the dilution of LDH that could be optimally read, various concentrations of LDH were assessed and absorption measured for linearity of OD reading (Figure 30).

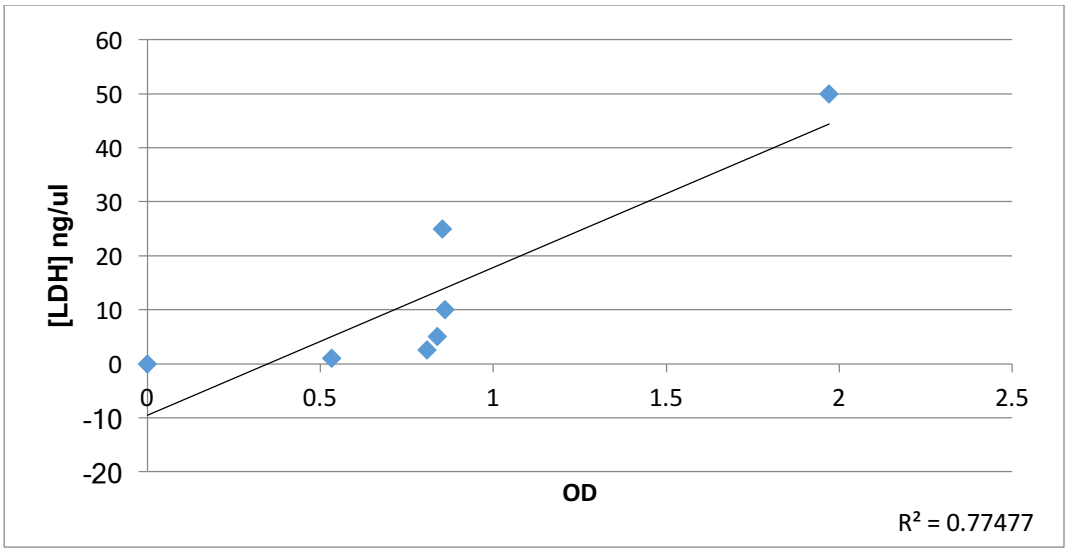


Figure 28. Standard curve for LDH Assay. In order to quantify the amount of LDH released by the cells, samples were compared to a standard curve of known LDH concentration and the corresponding OD reading detected by an absorbance plate reader. R-squared indicates how closely the data are to the fitted regression line plotted here.

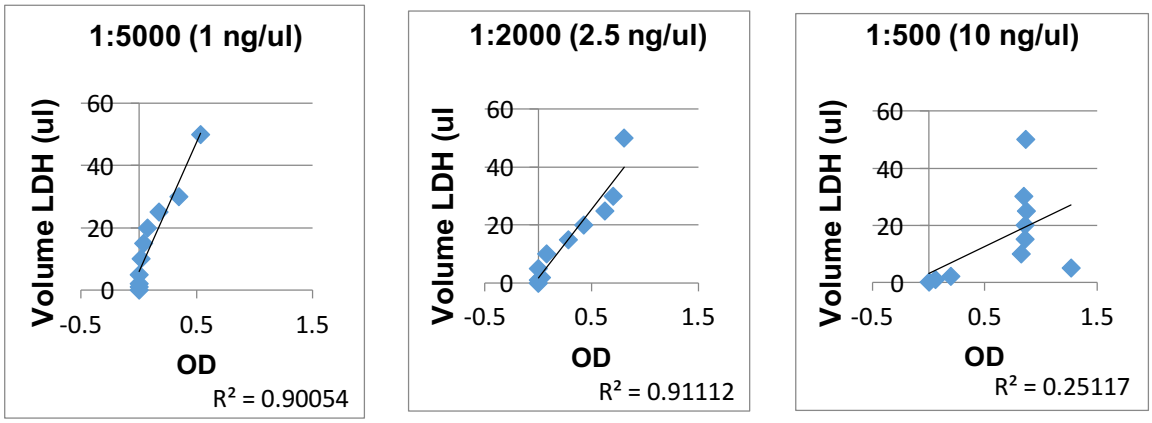


Figure 29. Increasing concentrations of LDH were assessed and absorption measured for linearity of OD reading. Concentrations of 1 ng/μL and 2.5 ng/μL produce readings that are close to the fitted regression line with an R^2 close to 1, while a concentration as high as 10 ng/μL does not.

In addition to optimizing the volume of conditioned media to use for measurement, it was imperative to understand the number of cells that lead to a particular reading within the linear range of the assay. An increasing number of HeLa cells produce a correlative amount of LDH that is reflected in the measurement detected by absorbance reading (Figure 31). An additional control experiment was performed to understand the expected OD reading from increasing numbers of injured and dying cells. HeLa cells were plated in a 48-well plate at increasing cell densities and cultured for 24 h (Figure 32). Triton-X was added to some wells (n=3) (Figure 32a) and remaining wells (n=3) were left untreated (Figure 32b). Addition of triton-X to HeLa cells increases the OD reading correlating to the amount of LDH released from cells into conditioned media.

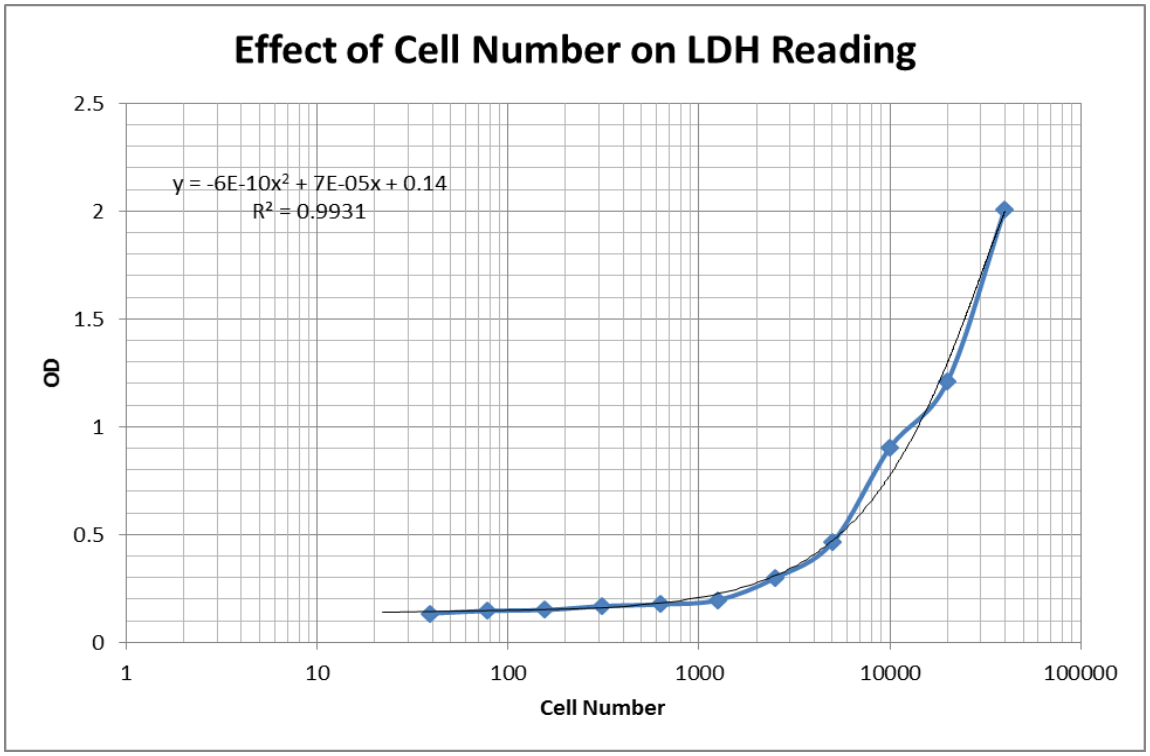


Figure 30. Cell number vs. optical depth of the LDH assay. An increasing number of cells produce a correlative amount of LDH that is reflected in the measurement detected by absorbance reading.

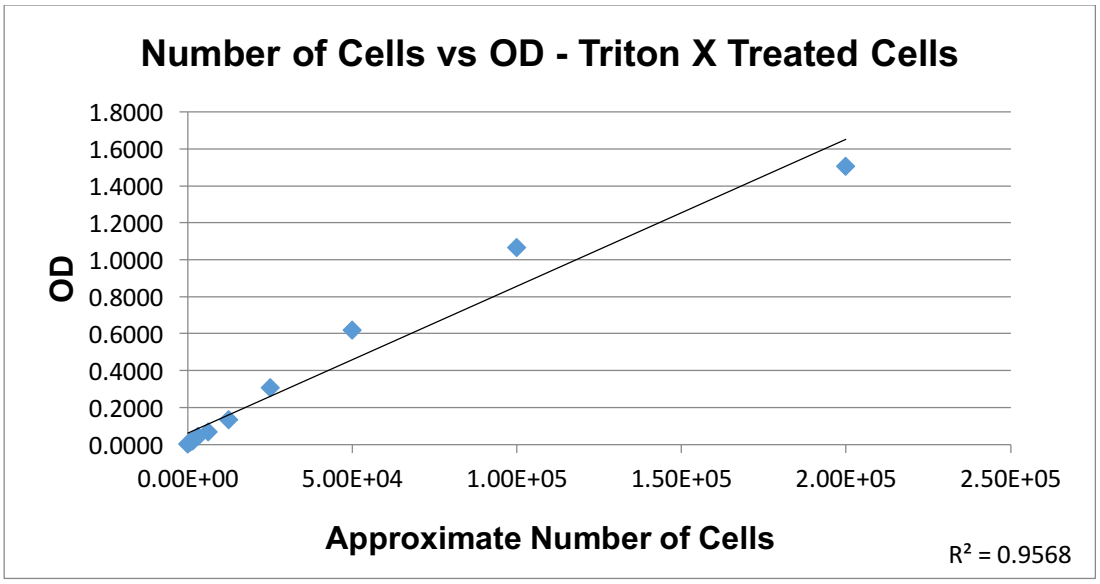


Figure 31a. . Increasing numbers of cells produce correlative increased amounts of LDH when treated with Triton-X as measured by absorbance.

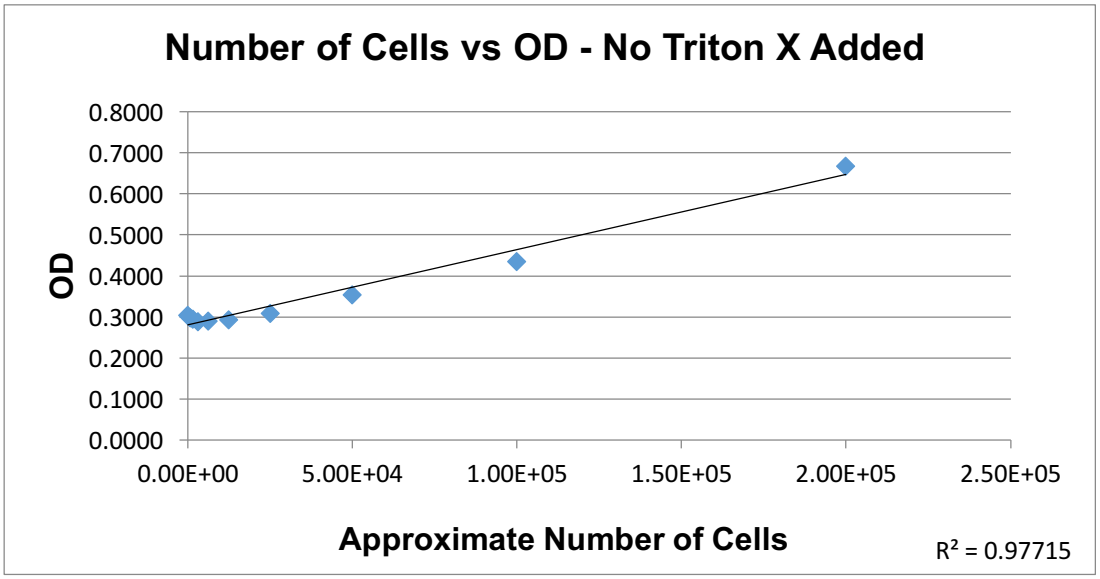


Figure 31b. Increasing numbers of cells produce correlative increased amounts of LDH as measured by absorbance. These readings are lower than those of cells treated with the detergent Triton-X, which damages the cell membrane.

D. siRNA Transfection

Cultured cells were grown in 24- or 48-well plates and transfected using Lipofectamine RNAiMax lipid-based transfection reagent (Life Technologies) per the manufacturer protocol. Transfection complexes were made using 50 µL per well Opti-MEM media (Gibco). Mock transfected cells contained Opti-MEM and 75 µL per well RNAiMax with no RNA. Cells were transfected for 48 h at 37ºC. Cells were then gently washed with PBS and lysed with RIPA buffer containing protease inhibitor. Protein estimation was performed using the BSA Protein Assay kit (Thermo).

E. Transcription Factor Activation Reporter Arrays

i. LucPhoto TransActivation Array

One-Step LucPhoto TransActivation Array was obtained from Signosis (Santa Clara, CA) and used according to the manufacturer protocol. Briefly, four sets of 24 baculovirus recombinant viruses (30 µL) were transferred into three columns of cell culture wells of a 96-well plate containing cells at 70% confluency and incubated for 24 h. MTM or TA was added to cells for 36 h to induce luciferase activity. An equal volume of CellTiter-Fluor Reagent (Promega) was added to add wells to measure cell viability. Plate was allowed to incubate for 30min at 37ºC. Fluorescence was measured at 360_{EX} 535_{EM}. Media was removed and cells were washed with PBS. PBS was removed and 20 µL passive lysis buffer was added to each well for a 15 min incubation period at room temperature. Luciferase substrate (100 µL) was added to each well and

mixed. Plate was immediately read in a Glomax luminometer (Promega) and reported as relative light units (RLUs).

ii. Transcription Factor Activation Profiling Array

Transcription factor activation profiling plates were obtained from Signosis (Santa Clara, CA) and used according to manufacturer protocol (Figure 40a). Each well of the plate was specifically pre-coated with complementary sequences of probes. Differentiated SK-N-SH cells were treated with vehicle, MTM, MTM-SDK, MTM-SK, TA, and the combination of MTM with TA in 100 mm dishes for 48 h. Nuclear protein extraction was performed. Biotin-labeled probes that are made based on consensus sequences of TF DNA-binding sites were incubated with nuclear extracts. Individual probes found the corresponding TF and formed TF/probe complexes. Bound probes were then detached from the complex and analyzed through hybridization with the plate. Captured DNA probe was then detected with streptavidin-horseradish peroxidase. Luminescence was measured with a Glomax luminometer (Promega) and reported as relative light units (RLUs).

F. Epigenetic Enzyme Activity Assays

i. **DNMT Activity Assay**

Kit was obtained from Active Motif and used according to manufacturer protocol. Differentiated SK-N-SH cells were treated with vehicle, MTM, MTM-SDK, MTM-SK, TA, and the combination of MTM with TA in 100 mm dishes for 48 h. Nuclear protein extraction was performed and protein estimation was done with both the cytoplasmic and nuclear fractions. Pseudoreplicates were used to

measure cell viability. To calculate the activity of DNMTs, an average of the pseudoreplicate readings was taken. The average blank OD was subtracted from this average pseudoreplicate OD. This quantity was then divided by the amount of protein added to the reaction in µg multiplied by the incubation time in hours. This total was then multiplied by 1000 to give activity in OD/h/mg.

ii. HDAC Activity Assay

Kit was obtained from Active Motif and used according to manufacturer protocol. The assay uses a peptide substrate with an acetylated lysine group that can be deacetylated by HDAC enzymes. When it has been deacetylated, the lysine reacts with the provided developing solution to release a chromophore. Differentiated SK-N-SH cells were treated with vehicle, MTM, MTM-SDK, MTM-SK, TA, and the combination of MTM with TA in 100 mm dishes for 48 h. Nuclear protein extraction was performed and protein estimation was done. In short, all components were thawed on ice and reagents prepared according to protocol. Nuclear extract was used at a concentration of 3 µL and added to 27 µl assay buffer to bring the test sample volume to 30 µL total. HDAC assay buffer, HDAC substrate, and test sample were added to wells of the plate and incubated at 37°C for 30 min. HDAC reactions were stopped with the addition of developing solution, and then incubated for 10 min at room temperature. Standard curve was created to determine enzymatic activity (not shown). The total amount of protein added was calculated using protein concentration and volume used. Absorbance was read in a plate reader at 415 nm.

G. Incucyte ZOOM

Incucyte ZOOM technology allows real-time live microscopic imaging of cells in culture. There is direct access to microscopic imaging while pharmacological agents have been applied directly to the cells at varying concentrations in culture medium. This access allows for genetic manipulations via siRNA transfection and reporter assays that cannot be done in whole animal models. We expect that optimization of Incucyte Zoom technology with post-mitotic tissue culture models will be a powerful contribution to the field of AD study. Plating conditions of the Incucyte Zoom were done using PC12 cells differentiated by NGF (Figure 33).

IV. Aim 1: Mechanistic study of the activation of Alzheimer's disease relevant transcription factors in human cells

A. Introduction

Transcription factors have been considered as possible therapeutic targets for years. Hesitation for use of TFs as drug targets is partially due to the fact that the interacting surface between DNA and TFs is quite large and subject to many changes during DNA-binding [143]. As previously discussed, SP1 has been shown to be relevant in neurodegenerative disorders, including AD. Mechanistic investigations of compounds thought to target SP1 may lead to a better understanding of how these compounds affect amyloid-beta pathway proteins. It is also important to understand whether these drugs act solely through SP1 or if in fact multiple TFs are involved in a pathway leading to changes in APP, BACE1, and Aβ.

B. Prediction Tools & Literature Review

A predicted gene network of SP1 with genes of the Aβ pathway was analyzed according to GeneMANIA (http://www.genemania.org August 2016 Assembly). AD-associated genes predicted to directly or indirectly interact with SP1 and other TFs were shown (Figure 33). Connections include genetic and physical interactions, co-localization, and co-expression. For example, ATF6 (Activating transcription factor 6) showed a genetic interaction with SP1. ATF6 has been implicated in the accumulation of misfolded proteins in diseases such as AD [144]. Brains from deceased individuals with Down syndrome were examined and up-regulation of E2F-1 was observed, exhibiting similar

neuropathological features of dementia of Alzheimer's type [145]. E2F-1 shares a pathway with SP1 and co-localizes with SP1 according to the gene prediction tool (Figure 33). Additionally, subcellular distribution of E2F-1 has been shown to be altered during AD [146]. The angiogenic TF Ets-1 was observed to be differentially expressed in AD brain versus control brains. Control brains showed little expression of Ets-1 whereas Ets-1 was ubiquitously expressed in the cortex and hippocampus of AD brain tissue [147]. Prediction tools along with an extensive literature review led to a working list of genes and TFs implicated in neurodegeneration and AD (Table 3).

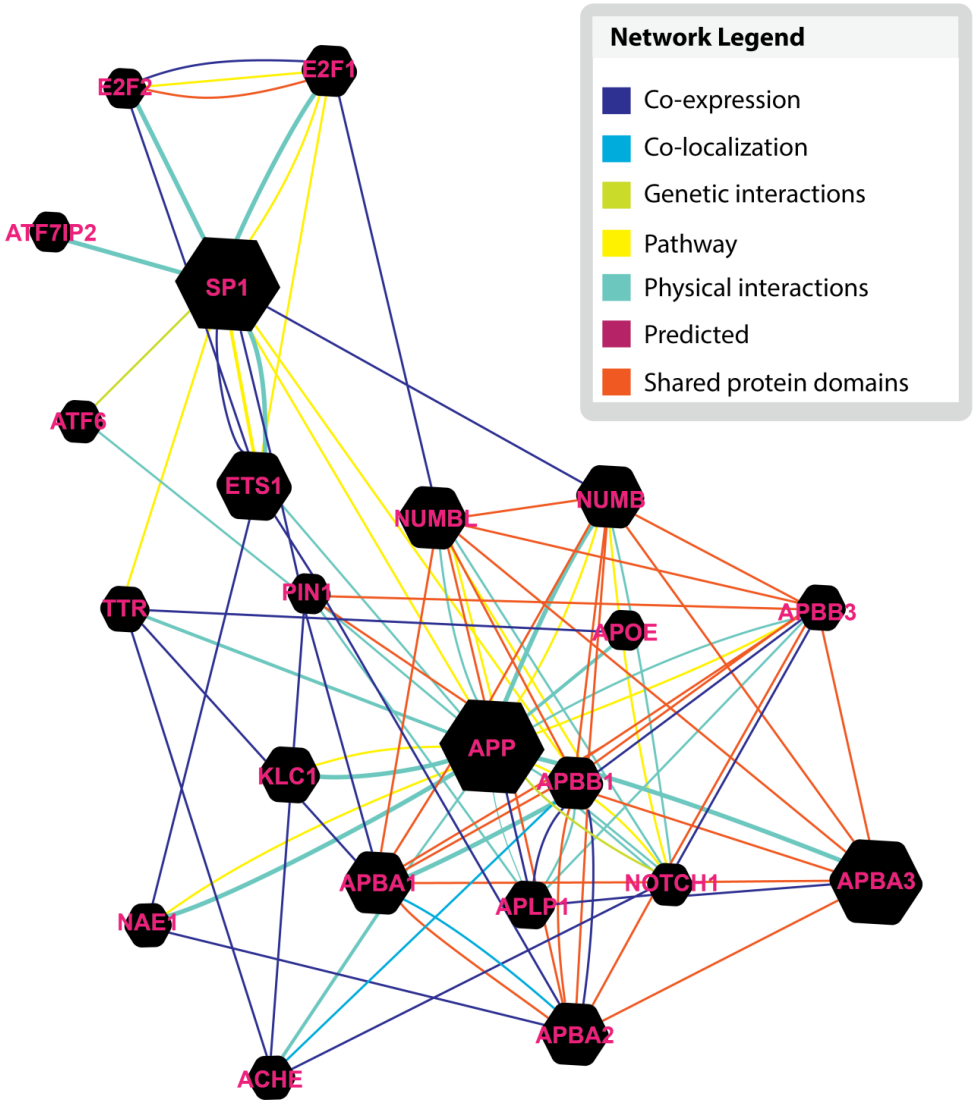


Figure 32. Predicted gene network of *SP1* and APP. Connections include genetic and physical interactions, co-localization, and co-expression. Many TFs of interest can be generated from prediction tools (GeneMANIA.org).

Table 3. Genes related to Alzheimer's disease by literature review

Name	Full Name	Aliases	Role of Protein	Relevance to AD
APBA1	amyloid beta (A4) precursor protein-binding, family A, member 1	MINT1, X11A, adapter protein X11alpha, D9S411E, LIN10 (GeneCards, Sept 2016)	Neuronal adaptor protein; involved in signal transduction; synaptic vesicle exocytosis by binding to Munc18-1 (GeneCards, Sept 2016)	Stabilizes APP; inhibits production of proteolytic APP fragments (RefSeq, Jul 2008)
APBB1	amyloid beta (A4) precursor protein-binding, family B, member 1	RIR, FE65 (GeneCards, Sept 2016)	Adaptor protein; located in the nucleus; transcription coregulator; binds modified histones (UniProtKB, Sept 2016)	Forms transctriptionally active complex with gamma-secretase-derived APP intracellular domain (Entrez, Sept 2016)
APP	amyloid beta (A4) precursor protein	AD1, CVAP, Peptidase Nexin, AAA (GeneCards, Sept 2016)	Cell surface receptor; involved in neurite growth, neuronal adhesion, axonogenesis and cell mobility (UniProtKB, Sept 2016)	Mutations in APP have been implicated in autosomal dominant AD; progressive cleavage can lead to amyloid-beta production (Tocris, Sept 2016)
ATF6	activating transcription factor 6	ATF6A (GeneCards, Sept 2016)	Transcription factor; activates target genes for the unfolded protein response during ER stress (Entrez, Sept 2016)	Neuronal death in AD may arise from ER dysfunction (Katayama et al., 2004)
CREB	cAMP response element-binding protein	CREB-1	Transcription factor; binds to cAMP response elements (CRE) [148]	Critical role in memory consolidation [149]

E2F1	E2F transcription factor 1	RBBP3, RBAP1, PBR3 (GeneCards, Sept 2016)	Transcription activator; binds DNA; mediates cell proliferation and TP53-dependent apoptosis (UniProtKB, Sept 2016)	E2F1 mediates death of amyloid-beta-treated cortical neurons (Jordan-Sciutto et al., 2001)
ETS1	v-ets avian erythroblastosis virus E26 oncogene homolog 1	EWSR2 (GeneCards, Sept 2016)	Transcription factor; controls expression of various cytokine and chemokines (UniProtKB, Sept 2016)	Differentially expressed in AD brain versus controls [147]
HIF1	hypoxia-inducible factor-1	HIF1; MOP1; PASD8; HIF-1A; bHLHe78; HIF-1alpha (GeneCards Mar 2017)	Respond to decreases in available oxygen in the cellular environment [150]	Stabilization of HIF1 levels is neuroprotective [151]
IDE	insulin degrading enzyme	Abeta-Degrading Protease, insulin (GeneCards, Sept 2016)	Zinc metallopeptidase; degrades insulin (Entrez, Sept 2016)	Degrades amyloid formed by APP; Deficiencies are associated with AD (UniProtKB, Sept 2014)
IL1A	interleukin 1 alpha	IL1, IL1F1 (GeneCards, Sept 2016)	Cytokine involved in various immune responses (Entrez, Sept 2016)	Induces synthesis and processing of APP (Griffin et al., 1995)
MME	membrane metallo-endopeptidase	CALLA, NEP, neprilysin (GeneCards, Sept 2016)	Acute lymphocytic leukemia antigen; neutral endopeptidase (Entrez, Sept 2016)	Amyloid-beta degrading enzyme ; regulation of neuropeptide signaling; levels are lower in AD brains (Akiyama et al., 2001)
NCSTN	nicastrin	ATAG1874, KIAA0253 (GeneCards, Sept 2016)	Type I transmembrane glycoprotein ; component of gamma-secretase complex (Entrez, Sept 2016)	Subunit of gamma-secretase which cleaves APP (UniProtKB, Sept 2014)

NFAT	nuclear factor of activated T-cells	NFAT1; NFATP; NFATC2 (GeneCards Jan 2017)	Induction of gene transcription during the immune response	Expressed by microglia which are stimulated by Aβ [152]
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells	p50; KBF1; p105; EBP-1; CVID12; NF-kB1 (GeneCards, Mar 2017)	Transcription regulator activated by various intra- and extra-cellular stimuli	Involved in Aβ-induced neuroinflammation [153]
PSEN1	presenilin 1	AD3, PS1, FAD, PSNL1 (GeneCards, Sept 2016)	Catalytic subunit of the gamma-secretase complex (UniProtKB, Sept 2016)	Patients with inherited AD carry mutations in PSEN1 or APP which lead to increased amyloid production (Entrez, Sept 2014)
SMAD		BSP1; JV41; BSP-1; JV4-1; MADH1; MADR1 (GeneCards Mar 2017)	signal transducers and transcriptional modulators that mediate multiple signaling pathways	Colocalizes with both neurofibrillary tangles and β-amyloid plaques [154, 155]
SP1	Specificity protein 1		involved in cell differentiation, cell growth, apoptosis, immune responses, response to DNA damage, and chromatin remodeling	Regulates expression of APP and BACE1 [78]
STAT1	signal transducer and activator of transcription 1	CANDF7; IMD31A; IMD31B; IMD31C; ISGF-3; STAT91 (GeneCards, Mar 2017)	activated by various ligands; mediates the expression of a variety of genes	Binding sites present on the APP promoter [156]

TNF	tumor necrosis factor	TNFA, TNFSF2 (GeneCards, Sept 2016)	Proinflammatory cytokine; involved in regulation of cell proliferation, differentiation, apoptosis (Entez, Sept 2016)	Neuroprotective in mice (RefSeq, Jul 2008); located near a chromosomal region (~30 Mb on 6p21) that has shown genetic linkage and association with AD (Bertram and Tanzi, 2004)
TTR	transthyretin	PALB, CTS1 (GeneCards, Sept 2016)	A CSF protein; Carrier protein; transports thyroid hormones (Entez, Sept 2016)	Lower concentrations of TTR in AD patients versus controls (Serot 2016)

C. Association Analysis

The genetic data in the Alzheimer's Disease Neuroimaging Initiative (ADNI) dataset, initiated in 2003 by the National Institute of Aging (NIA) has provided a source for quantitative endophenotype association studies [157]. A goal of ADNI studies has been to establish magnetic resonance imaging (MRI) and positron emission tomography (PET) imaging measures, as well as additional biomarkers from blood and cerebrospinal fluid (CSF), to influence eventual design of AD clinical trials [157]. We chose single nucleotide polymorphisms (SNPs) within the *SP1* gene from the ADNI genome wide association study (GWAS) data and performed an association analysis with an AD-specific imaging biomarker (entorhinal cortex thickness). We identified a significant SNP (rs11170553) associated with entorhinal cortex (ERC) thickness. The ERC is known to be affected in early AD. It has been shown that AD subjects with thinner ERC demonstrate lower baseline cognitive scores and higher disease severity [158] and that reduction of ERC thickness of MCI patients can predict decline over time [159]. SNP rs11170553 was also associated with cerebral amyloid deposition (Figure 34).

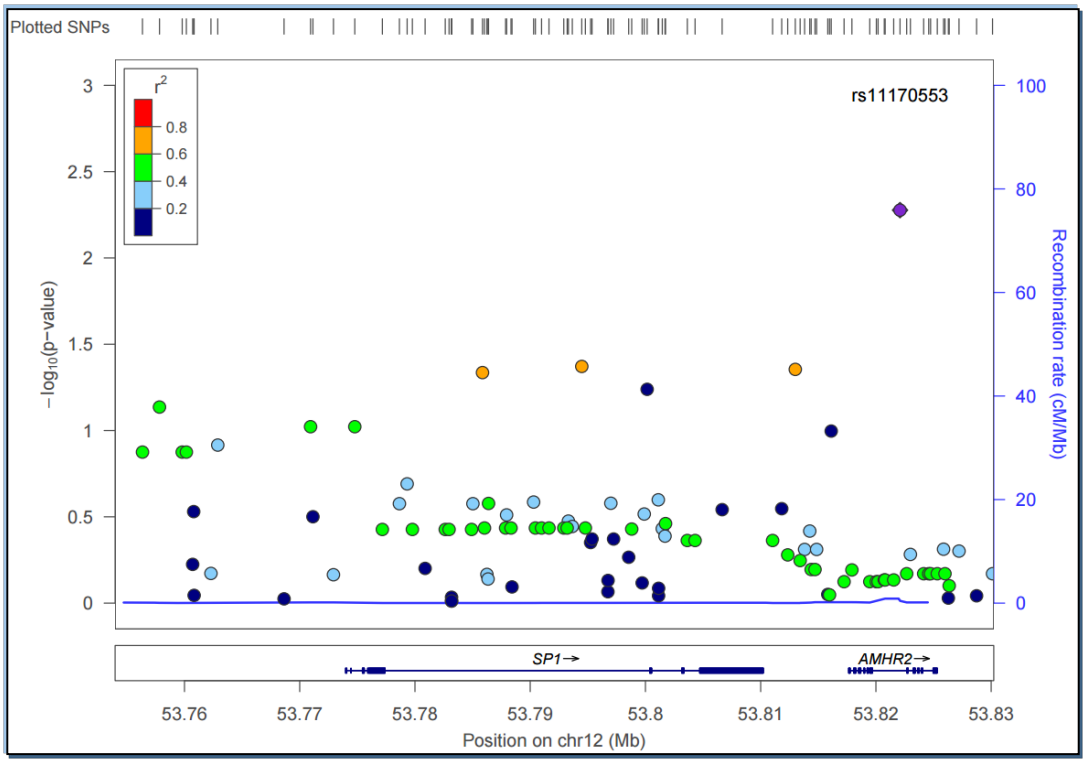


Figure 33. Association results of SNPs in *SP1* with an AD-specific neuroimaging phenotype (entorhinal cortex thickness). Single nucleotide polymorphisms (SNPs) within ±20 kb of the SP1 gene were used and rs11170553 was significantly associated with entorhinal cortex thickness after multiple comparison adjustment. Source: ADNI dataset

D. Results

i. Human Fetal Neurons

To measure the activation of multiple TFs in human neurons, HFN were transduced with recombinant viruses containing a particular TF (Figure 35). Four sets of 24 baculovirus recombinant viruses (30 µL) were transferred into three columns of cell culture wells of a 96-well plate containing cells at 70% confluency and incubated for 24 h. MTM was added to cells for 36 h to induce luciferase activity (Signosis, Inc.). Conditioned media was removed and placed in a new 96-well plate for cell toxicity to be measured by LDH assay. Luciferase reporter activity was read by a luminometer.

a. Effect of mithramycin A on cell toxicity of HFN

Cells treated with MTM (5 µM) changed cytotoxicity variably based on which TF construct was transduced into corresponding wells (Figure 36). Only cells transduced with AP1, PPARγ, and HIF1 corresponded to a decrease in cytotoxicity after treatment with MTM. SMAD-transduced cells and the CMV control showed no change in cell toxicity after MTM treatment.

b. Effect of mithramycin A on transcription factor activation of HFN

MTM decreased activation of few TFs in HFN (Figure 37). Notably, NFκB, NFAT, NRF2/ARE were decreased in luciferase activity by treatment with MTM. SP1 activation between vehicle and MTM treated cells was not different. Treatment with MTM increased activation of SMAD by nearly two-fold.

1	2	3	4	5	6	7	8	9	10	11	12
NFkB	STAT1	IRF	NFkB	STAT1	IRF	NFkB	STAT1	IRF	NFkB	STAT1	IRF
NFAT	YY1	SP1	NFAT	YY1	SP1	NFAT	YY1	SP1	NFAT	YY1	SP1
TCF/LEF	HSF	PR	TCF/LEF	HSF	PR	TCF/LEF	HSF	PR	TCF/LEF	HSF	PR
ATF6	NRF2	CREB	ATF6	NRF2	CREB	ATF6	NRF2	CREB	ATF6	NRF2	CREB
GAS/ISRE	XBP1	C/EBP	GAS/ISRE	XBP1	C/EBP	GAS/ISRE	XBP1	C/EBP	GAS/ISRE	XBP1	C/EBP
ER	PPARg	SRF	ER	PPARg	SRF	ER	PPARg	SRF	ER	PPARg	SRF
AP1	HIF1	Gli	AP1	HIF1	Gli	AP1	HIF1	Gli	AP1	HIF1	Gli
SMAD	AR	CMV	SMAD	AR	CMV	SMAD	AR	CMV	SMAD	AR	CMV

Figure 34. Transcription Factor Activation Profiling in neurons. Plate map of transcription factor. 3x 8 well strip, 4 reactions per TF. 24 recombinant viruses generated from insect cells with a modified baculovirus system that contains mammalian delivery element, TF consensus sequence, and luciferase reporter gene. Source: Signosis, Inc.

Figure 35. Effect of Mithramycin A treatment on human fetal neuron cytotoxicity. Cells treated with MTM (5 μM) changed cytotoxicity variably based on which TF construct was transduced into corresponding wells. Cells transduced with AP1, PPARγ, and HIF1 corresponded to a decrease in cytotoxicity after treatment with MTM. SMAD-transduced cells and the CMV control showed no change in cell toxicity after MTM treatment.

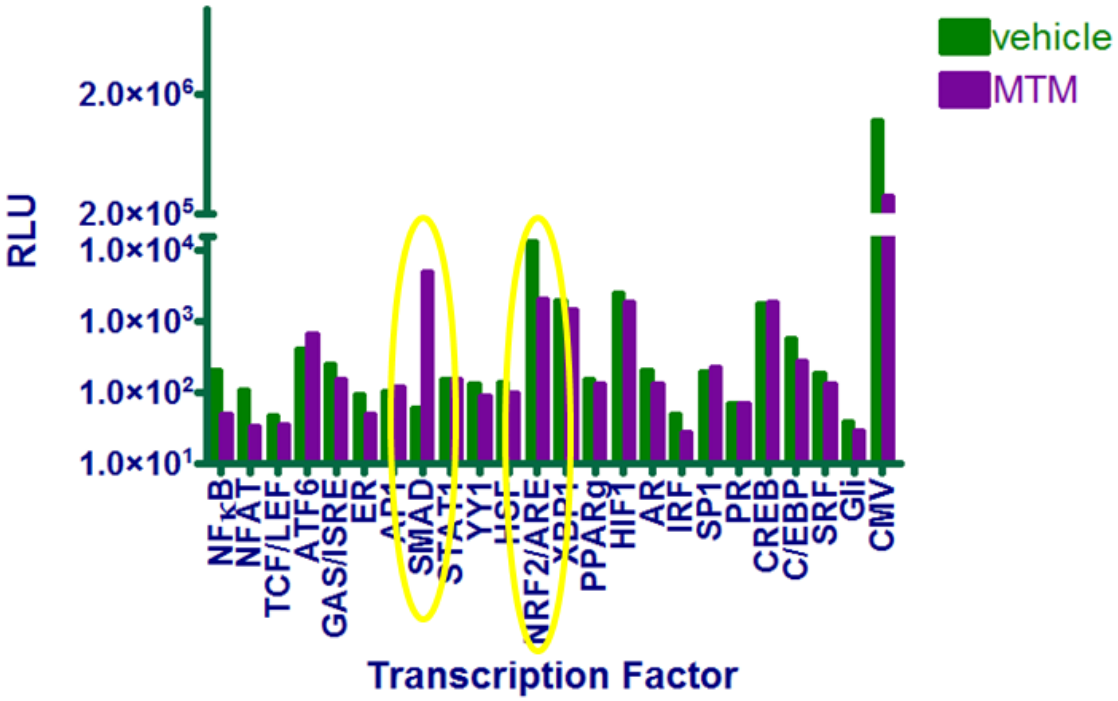


Figure 36. Effect of MTM on luciferase expression of various transcription factors in human fetal neurons. MTM decreased activation of few TFs. In particular, NFκB, NFAT, NRF2/ARE were decreased in luciferase activity by treatment with MTM. SP1 activation between vehicle and MTM treated cells was not different. Treatment with MTM increased activation of SMAD by nearly two-fold.

ii. Differentiated Neuroblastoma Cells

To measure the activation of multiple TFs and elucidate the status of signaling pathways after MTM or TA treatment, a TF reporter assay was performed in differentiated human neuroblastoma cells. First, were transduced with recombinant viruses as described earlier (Figure 35). Four sets of 24 baculovirus recombinant viruses (30 µL) were transferred into three columns of cell culture wells of a 96-well plate containing cells at 70% confluency and incubated for 24 h. MTM or TA was added to cells for 36 h to induce luciferase activity (Signosis, Inc.). Conditioned media was removed and placed in a new 96-well plate for cell toxicity to be measured by LDH assay. Luciferase reporter activity was read by luminometer.

a. Effect of mithramycin A on cell toxicity of NBRA

Neurons transduced with IRF, SP1, and SRF showed increased cytotoxicity compared to vehicle treatment (Figure 38a). YY1, NRF2/ARE, and XPB1 transduced cells showed a decrease in cell toxicity. Others showed no change in toxicity as compared to vehicle.

b. Effect of mithramycin A on transcription factor activation of NBRA

MTM treatment led to nearly 80% decrease in SP1 reporter activity compared to vehicle treatment (Figure 38b). Several other TFs were decreased in activity by MTM treatment, including NFκB, NFAT, ER, AP1, and HIF1.

c. Effect of tolfenamic acid on cell toxicity of NBRA

Neurons transduced with XBP1 showed decreased cytotoxicity as detected by LDH assay (Figure 39a). Transduction by other TFs was well tolerated with and without TA treatment.

d. Effect of tolfenamic acid on transcription factor activation of NBRA

TA treatment led to no change in SP1 reporter activity (Figure 39b). Several TFs showed no change in activation after treatment with TA. However, NFκB was shown to be activated by TA treatment in NBRA.

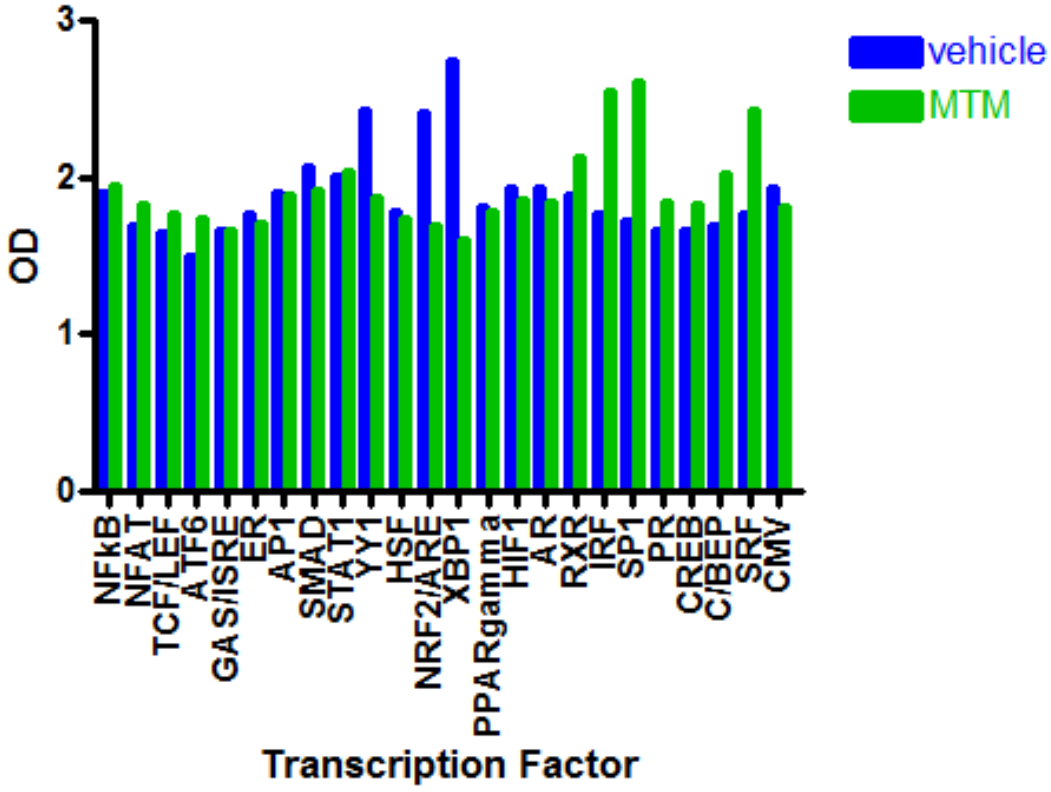


Figure 37a. Effect of MTM on cell toxicity in differentiated neuroblastoma cells as measured by LDH assay. Neurons transduced with IRF, SP1, and SRF showed increased cytotoxicity compared to vehicle treatment. YY1, NRF2/ARE, and XPB1 transduced cells showed a decrease in cell toxicity. Others showed no change in toxicity as compared to vehicle.

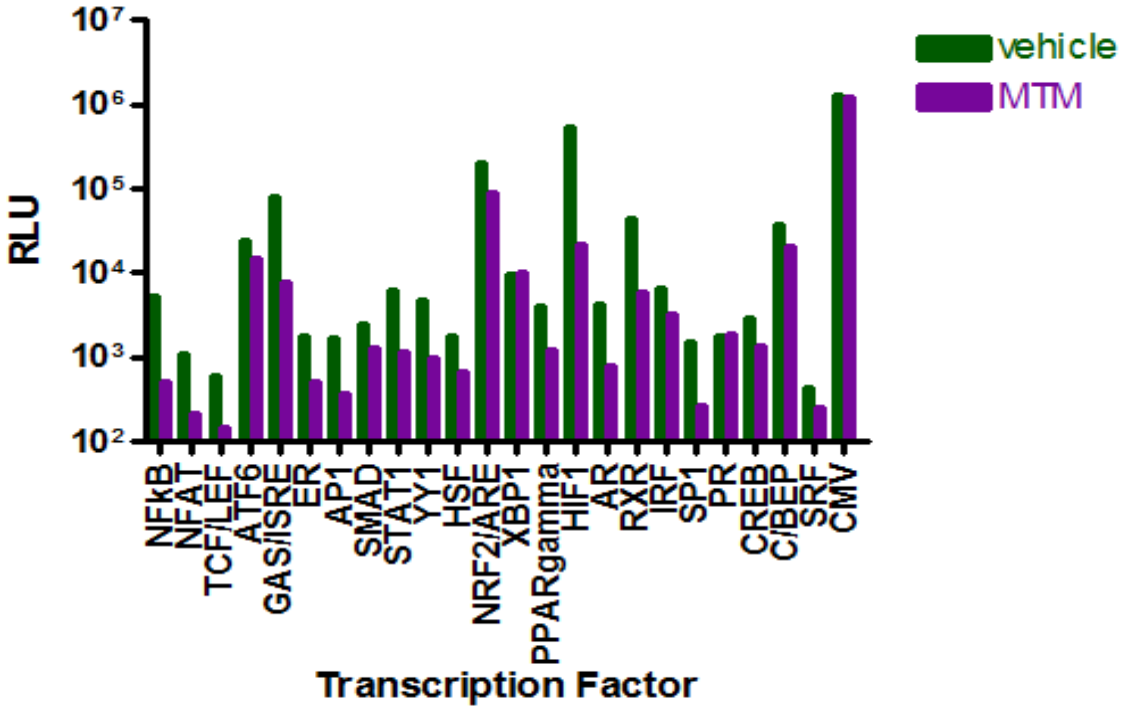


Figure 37b. Effect of MTM on luciferase expression of various transcription factors in differentiated neuroblastoma cells. Four sets of 24 baculovirus recombinant viruses (30 µL) were transferred into three columns of cell culture wells of a 96-well plate containing cells at 70% confluency and incubated for 24 h. MTM or TA was added to cells for 36 h to induce luciferase activity. MTM leads to nearly 80% decrease in SP1 reporter activity.

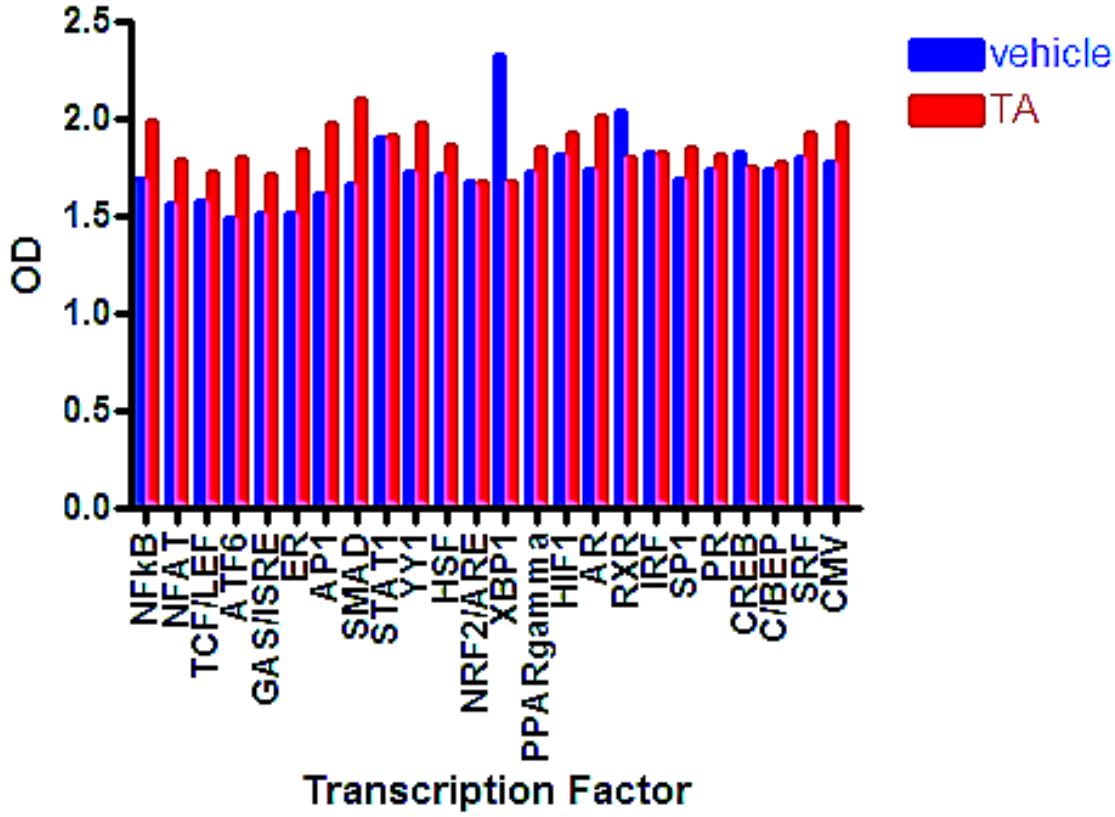


Figure 38a. Effect of TA on cell toxicity in differentiated neuroblastoma cells as measured by LDH assay. Neurons transduced with XBP1 showed decreased cytotoxicity as detected by LDH assay. Transduction by other TFs was well tolerated with and without TA treatment.

Figure 38b. Effect of TA on luciferase expression of various transcription factors in differentiated neuroblastoma cells. Four sets of 24 baculovirus recombinant viruses (30 µL) were transferred into three columns of cell culture wells of a 96-well plate containing cells at 70% confluency and incubated for 24 h. MTM or TA was added to cells for 36 h to induce luciferase activitySeveral TFs showed no change in activation after treatment with TA, including SP1. However, NFκB was shown to be activated by TA treatment in NBRA.

e. Effect of mithramycin A and tolfenamic acid on transcription factor activation of NBRA

In order to directly assess the differences in TF activation between MTM and TA, a TF profile array was performed as depicted in Figure 40a. In this experiment, 48 TFs were examined in NBRA treated with either MTM or TA (Figure 40b). Several TFs including Brn-3, CAR, HIF, NFE2, and p53 show a 10-fold change or higher in activation in MTM vs TA treated neurons. FAST-1, IRF, and STAT5 all show a 5-fold change or higher.

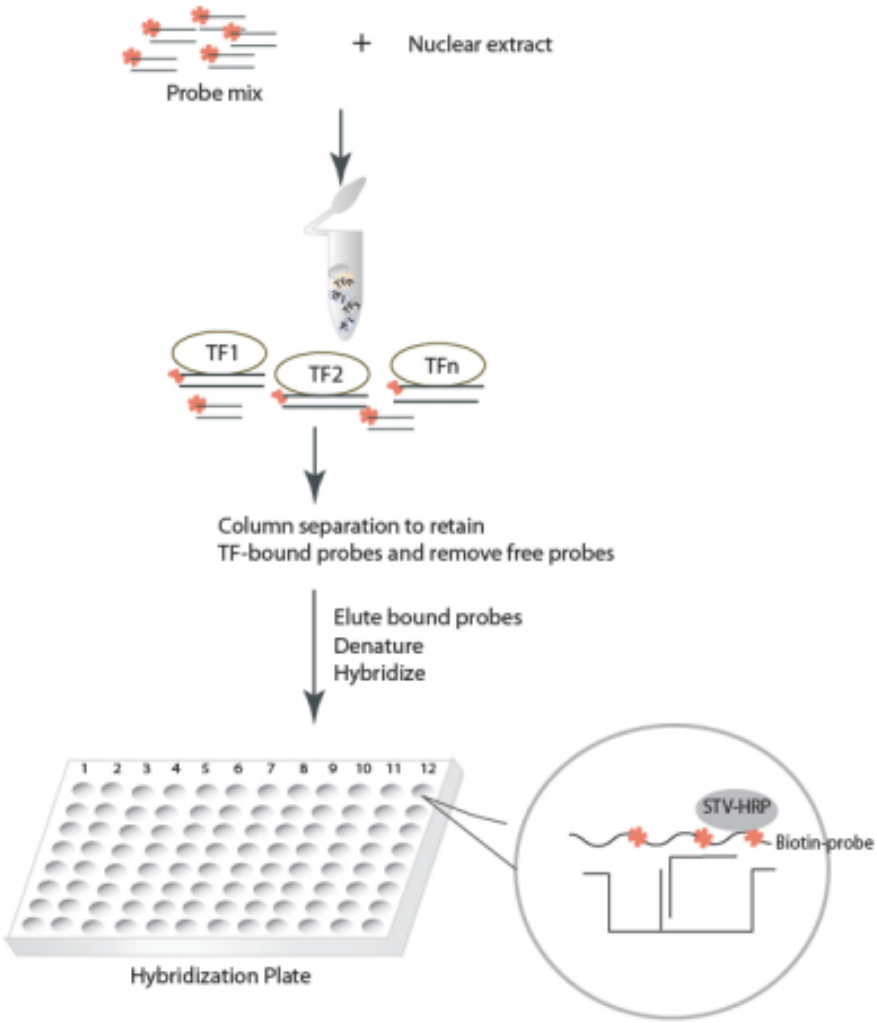


Figure 39a. Transcription factor profile array. Biotin-labeled probes that are made based on consensus sequences of TF DNA-binding sites were incubated with nuclear extracts. Individual probes found the corresponding TF and formed TF/probe complexes. Bound probes were then detached from the complex and analyzed through hybridization with the plate. Captured DNA probe was then detected with streptavidin-horseradish peroxidase. (Figure from Signosis Inc.)

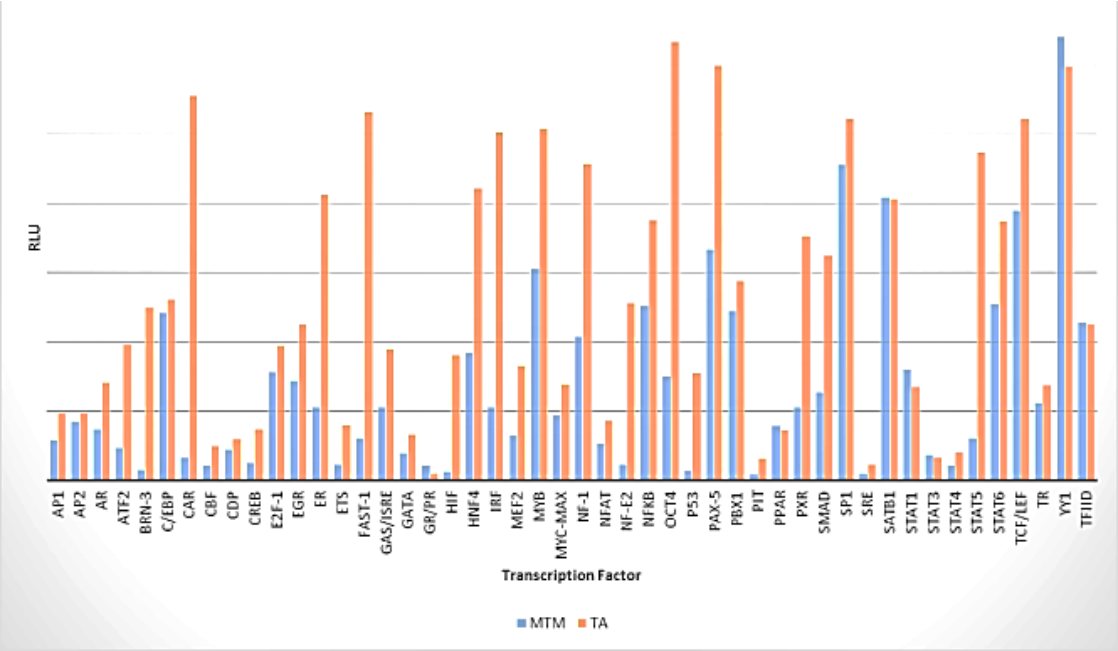


Figure 39b. Transcription factor profile array. An array of various TFs shown on the x-axis with the corresponding activation after treatment with MTM or TA measured by relative luminescence units (RLU) on the y-axis. Several TFs including Brn-3, CAR, HIF, NFE2, and p53 show a 10-fold change or higher in activation in MTM vs TA treated neurons.

E. Discussion

Treatment of a mixed brain cell culture system such as HFN with MTM yielded unexpected results in the activation of various TFs (Figure 37). Although SP1 activity was decreased by treatment with MTM in NBRA, it is unchanged in HFN. In a culture solely comprised of mature neurons, treatment with MTM decreases SP1 reporter activity as hypothesized. The effect of glia and other cells in a mixed brain culture on the activation of TFs could be a cause of these differences. For instance, it is known that particular cells of the CNS express certain TFs. Microglia express the pro-inflammatory transcription factor, NFAT [152]. Neurons may secrete factors which induce the expression of TFs by astrocytes [160]. The influence of neurons on glia and vice versa in a mixed brain culture such as HFN may complicate the direct effects we see from a simple reporter assay. Nevertheless, the NBRA culture allows us to observe the effects of two so-called SP1-modulating drugs, MTM and TA in a purely neuronal model.

Treatment with MTM led to a decrease in NFκB activity, yet treatment with TA resulted in an increase. This trend was observed in several of the TFs also predicted to be relevant of AD. The transcription factor profile array allowed head to head comparison of the effects MTM and TA have on various TFs (Figure 40b). Along with SP1, several other TFs show a decrease in activation when treated with MTM and no change with TA treatment. TFs of interest show a decrease with MTM treatment and a corresponding decrease (or no change) with TA treatment, binding sites on AD-related gene promoters, or found in the

literature to be relevant to AD. Understanding how these drugs differentially modulate various TFs may help elucidate a mechanism of action for these compounds relevant to AD.

Transcription factor modulation by small compounds can be achieved by four main strategies [143]. The first strategy involves the inhibition of protein-protein interactions, since many TFs rely on co-factors in order to function appropriately. Blocking TF binding to DNA requires detained understanding about the binding surface of the ligand. Small, clearly defined protein surfaces are ideal. Directly targeting and manipulating the TF DNA binding domain (DMD) by small molecules to either change its conformation of prevent DNA binding is yet another approach. TA has been shown to promote the degradation of SP1 [161]. Another strategy involves the targeting of chromatin remodeling/epigenetic reader proteins, which are essential for the access of TFs to DNA access. The fourth approach is based on compounds that would compete with TFs to block protein-DNA binding or by altering the conformation of target DNA sequences such that they cannot be recognized by TFs. MTM is thought to compete with and displace SP1 from its binding sites. The work presented here indicates that not only do MTM and TA activate SP1 differently; they also activate several other TFs. This function speaks to their differences in mechanism of action and the possibility of multiple networks of TFs involved in their action on amyloid pathway genes.

There is great future potential in compounds that work by any of these approaches. An HSP60-binding compound has been created, which prevents

HIF1 activation, likely by HIF1α crosslinking [162]. Another compound, inS3-54 inhibits transcriptional activity of STAT3 [163]. Understanding the molecular mechanisms of TF activation and function will inevitably provide clues how modulating specific TF function by drugs such as MTM and TA can be achieved and possibly also applied for AD therapy.

V. Aim 2: Examination of the effects of transcription factor modulating compounds on epigenetic markers in human neurons

A. Introduction

Epigenetic mechanisms are involved in the regulation of processes related to memory consolidation, aging, and cognition during the normal lifespan [164]. Environmental factors may play a role in the interaction with specific loci by modifying their expression through epigenetic mechanisms to increase susceptibility to disorders later in life [80, 165, 166]. Lifestyle, genetic factors, and environmental exposures in early life may all influence the epigenome in a latent manner [167, 168]. Possible treatments could target particular pathways associated with dysfunction. DNA methylation, chromatin modifications, and even microRNA expression have all been shown to be altered in neurodegenerative disorders. DNA methylation takes place when a methyl group is added at CpG dinucleotides which can activate or repress the transcriptional activity of a gene [169]. Histone deacetylaces (HDACs) are involved in the regulation of histones and many neurological processes [170]. For example, DNA hypomethylation leads to expanded regulation of the TF NFκB [171]. Understanding how certain compounds influence methylation and/or histone modification is important for understanding how TF binding may be affected by them. The experiments described in this section sought to discover the effect treatment with SP1-modulating compounds would have on histone deaceylase activity or DNA methyltransferase (DNMT) activity in human cells. Differentiated SK-N-SH (NBRA) cells were treated with vehicle, MTM, MTM-SDK, MTM-SK,

TA, and the combination of MTM with TA in 100 mm dishes for 48 h. Nuclear protein extraction was then performed.

B. Results

i. Effect of drug treatment on SP1 expression levels in nuclear extract

Nuclear extracts from NBRA treated with compounds showed variation in SP1 protein levels via Western immunoblotting (Figure 41). GAPDH was used as a loading control and levels of SP1 for each sample were normalized to the corresponding GAPDH level for that sample. MTM treated neurons as well as those treated with combination MTM and TA showed a significant decrease in SP1 as compared to vehicle. Cells treated with analog MTM-SK and those treated with TA showed an increase in SP1 levels.

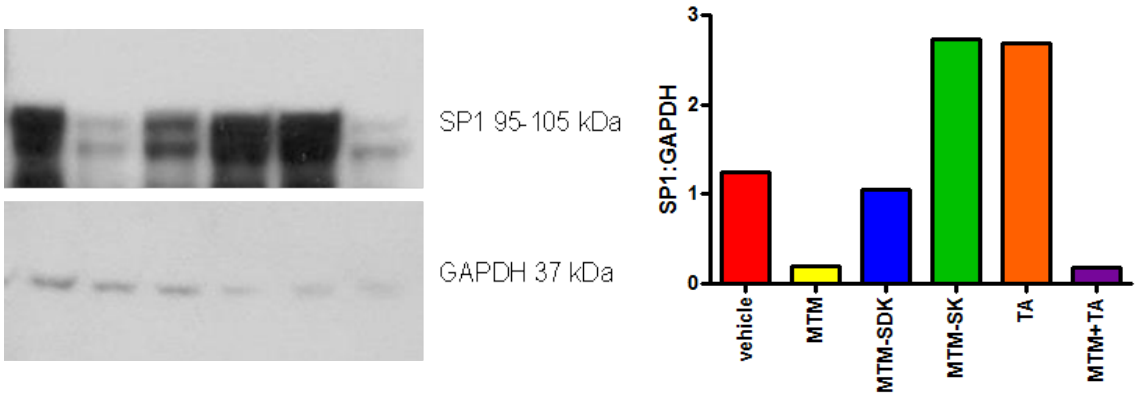


Figure 40. Effect of treatment of differentiated neuroblastoma cells with MTM, its analogs, and TA on levels of SP1. Nuclear extracts from NBRA treated with compounds show variation in SP1 levels. Lower levels were detected in the cytosolic fraction (not shown). MTM treated neurons as well as those treated with combination MTM and TA show a significant decrease in SP1 as compared to vehicle. Cells treated with analog MTM-SK and those treated with TA showed an increase in SP1 levels.

ii. Effect of drug treatment on DNMT activity

Retinoic acid-differentiated neurons (NBRA) were treated with compounds for 48 h. Cells were harvested and nuclear protein was extracted to measure DNMT activity. MTM-SDK significantly reduced DNMT activity in these neurons (Figure 42).

iii. Effect of drug treatment on HDAC activity

Neurons (NBRA) were treated with compounds for 48 h. Cells were harvested and nuclear protein was extracted to measure HDAC activity. MTM-SDK significantly reduced HDAC activity in neurons (Figure 43).

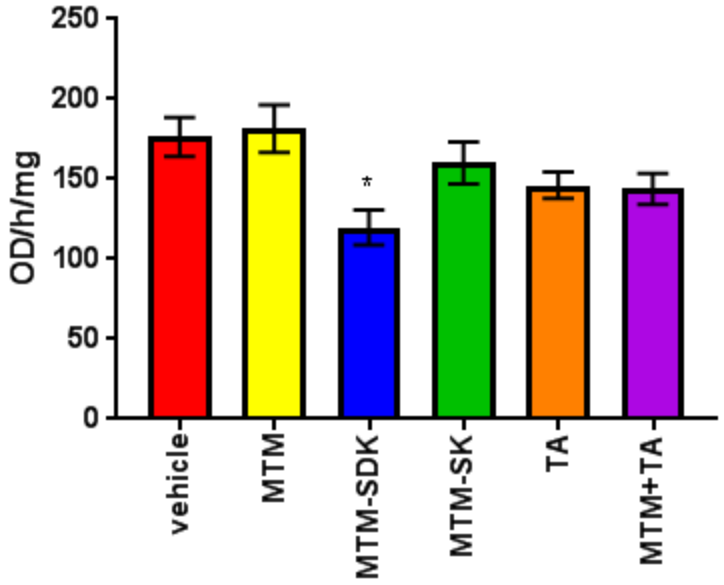


Figure 41. DNMT activity is significantly decreased in neurons treated with MTM-SDK. A sensitive ELISA-based enzyme activity assay using the high affinity binding of methyl CpG binding domain (MBD) protein towards methylated DNA to detect DNA methyltransferase activity on the provided CpG-enriched DNA substrate was used per manufacturer protocol. Methylated DNA was recognized by His-tagged MBD in amounts proportional to enzyme activity. An HRP-conjugated polyhistidine antibody provided the readout via spectrophotometer (OD/h/mg). Differentiated SK-N-SH cells were treated with vehicle, MTM, MTM-SDK, MTM-SK, TA, and the combination of MTM with TA in 100 mm dishes for 48 h. Nuclear protein extraction was performed and protein estimation was done with both the cytoplasmic and nuclear fractions. Pseudoreplicates were used (n=3). To calculate the activity of DNMTs, an average of the pseudoreplicate readings was taken. The average blank OD was subtracted from this average pseudoreplicate OD. This quantity was then

divided by the amount of protein added to the reaction in µg multiplied by the incubation time in hours. This total was then multiplied by 1000 to give activity in OD/h/mg.

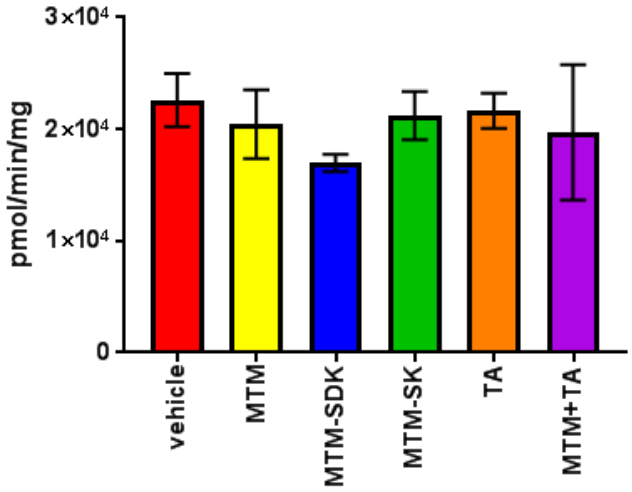


Figure 42. Treatment with SP1 modulating drugs does not significantly change histone deacetylase activity (HDAC) in neurons. The assay uses a peptide substrate with an acetylated lysine group that can be deacetylated by HDAC enzymes. When it has been deacetylated, the lysine reacts with the provided developing solution to release a chromophore. Differentiated SK-N-SH cells were treated with vehicle, MTM, MTM-SDK, MTM-SK, TA, and the combination of MTM with TA in 100 mm dishes for 48 h. Nuclear protein extraction was performed and protein estimation was done. Nuclear extract was used at a concentration of 3 µL and added to 27 µl assay buffer to bring the test sample volume to 30 µL total. HDAC assay buffer, HDAC substrate, and test sample were added to wells of the plate and incubated at 37°C for 30 min. HDAC reactions were stopped with the addition of developing solution, and then incubated for 10 min at room temperature. Standard curve was created to determine enzymatic activity (not shown). Absorbance was read in a plate reader at 415 nm.

C. Discussion

DNA methylation is a key epigenetic mechanism regulating gene expression. Understanding changes in the methylation state of the promoters of AD-relevant genes will provide insight into how to manipulate epigenetic modifications by various compounds. It is unknown if MTM-SDK treatment leads to hypomethylation of SP1 or possibly the promoter regions to which SP1 binds. It would also be imperative to understand whether this possible hypomethylation would enhance or inhibit SP1 or other TF binding. Moreover, understanding the cross-talk between TFs of interest and epigenetic programs will be critical in our understanding of how to manipulate these interactions and decrease amyloid-beta production. The effect MTM-SDK may have on the epigenetic state of the APP, BACE1, and other AD-relevant promoters may involve affecting how TFs such as SP1 bind and regulate their activity.

The idea of “undoing epigenetics” is a concept worth investigating as modifications that altered gene expression in early life may be reversible later in life {Weaver, 2005 #643}. DNMTs can be activated during both childhood and adulthood, and this plasticity can possibly be manipulated by compounds that target transcription regulators such as TFs.

Ultimately, a mechanism of epigenetic control involving the APP or BACE1 promoter, which potentially can be targeted by tailored small molecules toward TFs could be groundbreaking for AD research. The TFs themselves may not be the target, but the epigenetic regulation of the binding of that TF may serve a better approach. Elucidating the methylation status before and after treatment

with these compounds will be an important next step in evaluating the utility of MTM-SDK for AD. As DNA methylation status varies between cell type [172], it will be critical to test the effects of these drugs in other human cell culture models to discover any changes in methyltransferase and HDAC activity also. Association studies investigating a link between methylation status of AD-relevant promoters and AD-relevant phenotypes would also be valuable information to build a case for MTM or its analog MTM-SDK in AD therapy, as well as finding the roles of possible variants in altering TF binding. These epigenetic alterations are both dynamic and reversible, making these mechanisms attractive for future small compound discovery.

VI. Aim 3: Testing the effects of TF-modulating compounds on human glial, neuronal, and primary mixed brain cultures

A. Introduction

Cell lines derived from malignant brain tumors have been used in neuroscience studies for years. However, using primary brain and neuron cultures is more useful when trying to mimic the biological behavior of these cells and trying to avoid artifacts that may arise from using immortalized cells. Most neuronal cultures come from rodent brains, mostly because of their availability and cost [173]. Rodents do not, however, develop AD or other neurodegenerative diseases, which is a downside of these models. Use of human neurons from induced pluripotent stem cells and cultures from nonhuman primates are other more expensive options for models in AD study [142]. Recently, our laboratory was able to outline a protocol for the preparation and use of a human primary mixed brain culture derived from human fetal brain that has been successfully used in current work [142, 174]. Human fetal neurons are useful in AD studies, yet these cells, once plated, cannot be serially passaged or propagated for future experiments. For this reason, we have begun isolating free-floating cultures of neural stem cells from human fetal brain tissue (See Methods). These free-floating cultures are called neurospheres. In 1992, Reynolds and Weiss isolated and expanded undifferentiated neurospheres and were able to dissociate these cells to form more secondary spheres [175, 176]. These methods have been extensively used by research groups in numerous studies in animal models. However, this system is used for the production of

human neural cells in culture that can be frozen, stored, thawed, and cultured again to provide a supply of neurons with banking potential for future experimental use. These 3D neurospheres can also be induced to differentiate to form a monolayer of major CNS cell types using various growth factors [177-180]. We seek to further elucidate the identity and stemness of human NSPc. Our studies further characterized these differentiated cells and the conditions needed to optimize its use as a model for AD study.

In addition, use of the differentiated neuroblastoma cell line SK-N-SH (NBRA) as a post-mitotic human model allows us to easily manipulate neurons in a cancer cell line system. In contrast to the dNSPc model, NBRA are not a mixed population culture. Use of mature, differentiated neurons also permits the study of the neurobiology of these cells via real time live cell culture imaging with Incucyte Zoom technology (see Methods), assess amyloid beta levels via specific sandwich ELISA, and detect protein levels by Western immunoblotting.

B. Results

i. Drug dosage studies

Preliminary drug studies were performed in unique dividing cell lines to observe the effect of cell viability and toxicity across cell types. It was imperative to know if partial inhibition of BACE1 or APP via SP1 modulation could allow reduction of Aβ without mechanism-based toxicities from its action on other substrates. Also, use of purely neuronal (NBRA) and glial (U373) cell culture models provided insight into how these compounds affect particular cell types.

a. Human glioblastoma cells

The human glioblastoma cell line U373 was used in several preliminary studies along with NBRA to demonstrate which doses could be tolerated and used in future experiments. U373 were plated in 24-well plates at 70% confluency in MEM supplemented with 10% FBS and 1X antibiotic. Cells were treated with vehicle (0.01% DMSO), 0.05, 0.1, 0.5 1.0, and 10.0 μM MTM (Figure 44a) or vehicle (0.01% DMSO), 0.1, 1.0, 10.0, 50.0 and 100.0 μM TA (Figure 44b) for 16 h and 24 h. Cell toxicity was measured via LDH cytotoxicity assay as previously described at 2 time points. Treatment with increasing doses of MTM does not produce a dose-dependent increase in LDH production. At 16 h, higher doses in fact decrease cytotoxicity in U373. Although there are fluxations in LDH at increasing doses at the 24 h time point at the highest dose (10 μM), toxicity is 20% less than the lowest dose of MTM. Toxicity greatly increases at the 10 μM dose of TA at both time points in U373. Lower doses of 0.1 and 1.0 μM TA are well tolerated in U373.

To discover a possible dose-dependent effect of MTM on APP, U373 were treated with two non-toxic doses of drug (0.5 and 1.0 μM) for 48 h. Cells were harvested and Western immunoblotting was performed as previously described. GAPDH was used as a loading control and APP levels were normalized to it. APP levels are significantly decreased with MTM treatment, however, not in a dose dependent manner (Figure 46). Next, the effect of both MTM and TA on the levels of APP, SP1, and BACE1 in U373 was assessed via Western blot (Figure 47). U373 were plated in a 24-well plate at 70% confluency. Cells were

transfected with APP, BACE1, and SP1 siRNAs as controls (n=4). Other cells were treated with MTM or TA (1.0 or 5.0 μM). Beta-actin was used as the loading control in this instance. It was clear that both doses of MTM reduced APP levels in U373 and that TA caused no such change. It is notable here that the transfection with APP siRNA reduced APP expression as well, yet SP1 siRNA and BACE1 siRNA did not reduce their target proteins.

b. Human neuroblastoma cells

Combination treatments were used to compare how MTM and TA affect human cells separately and in concert. Increasing doses of MTM and TA in differentiation media were added to DIV7 NBRA. Doses from 0.05 to 50,000 nM were used to cover a wide range. Both cell viability and cell toxicity were assessed via CTG and LDH respectively (Figure 45). Combination treatment decreases viability by 30% and increases toxicity by 20% of vehicle at the dose of 5 nM. As expected, the toxicity and viability changes have an inverse relationship as peak toxicity (80% increase) is reached at 50 nM which corresponds to a 60% decrease in viability.

c. Human fetal neuron culture

The effect of these drugs on purely neuronal and glial cultures is important to understand, but ultimately we sought to understand their effect on the cell viability on primary mixed brain cultures. HFN cultures were plated in a 24-well format and treated with 6 doses (0, 1, 10,100, 500, 1000nM) of MTM, TA, MTM-SDK, MTM-SK, or combinations of MTM-SDK and TA or MTM-SK and TA for 48 h (Figure 48). MTM analog MTM-SDK did not show a change in cell viability even

at higher doses and in combination with TA. MTM-SK in combination with TA led to a significant decline in cell viability at the highest doses. Mixed brain cultures again showed that the primary cells of different populations affected the overall response to SP1 modulating drugs.

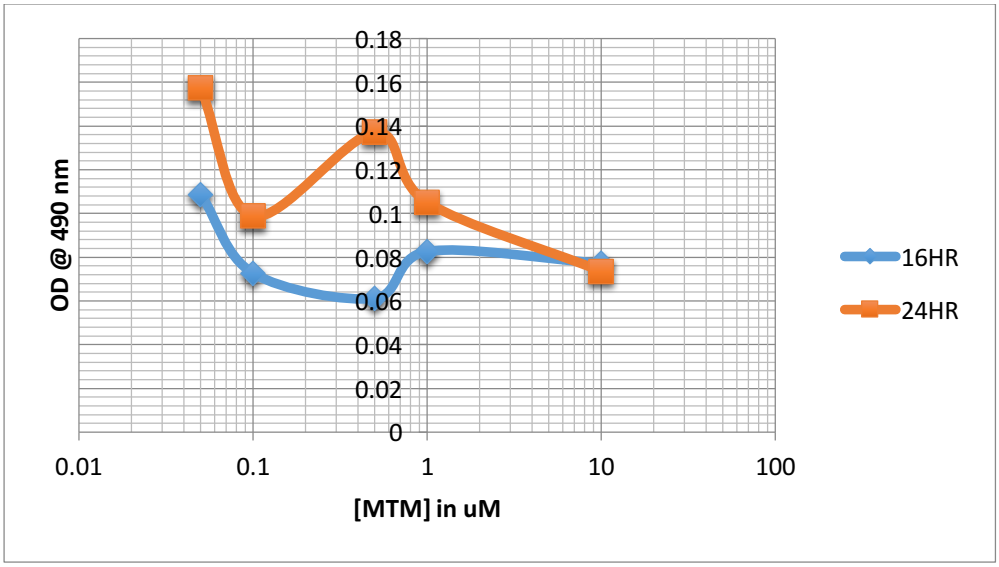


Figure 43a. Effect of MTM on cell toxicity in U373 as measured by the LDH assay. Cells were treated with vehicle (0.01% DMSO), 0.05, 0.1, 0.5 1.0, and 10.0 μM MTM for 16 h and 24 h. Treatment with increasing doses of MTM did not produce a dose-dependent increase in LDH production.

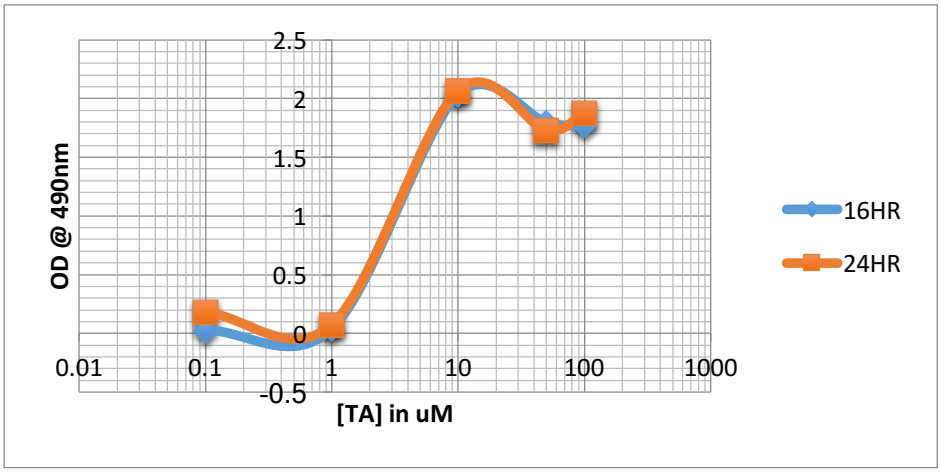


Figure 43b. Effect of TA on cell toxicity in U373 as measured by LDH assay. Cells were treated with vehicle (0.01% DMSO), 0.1, 1.0, 10.0, 50.0 and 100.0 μM TA for 16 h and 24 h. Toxicity greatly increased at the 10 μM dose. Lower doses of 0.1 and 1.0 μM TA were well tolerated in U373.

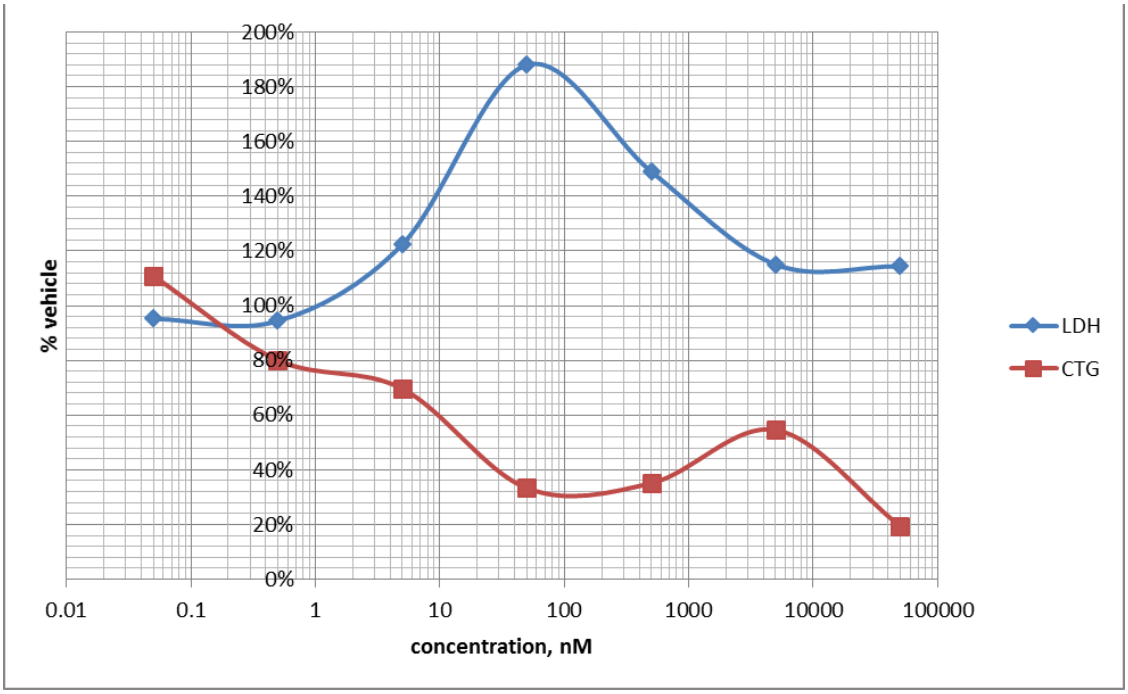


Figure 44. Effect of MTM and TA combination treatment on the viability (CTG) and toxicity (LDH) of NBRA. As expected, the toxicity and viability changes had an inverse relationship as peak toxicity (80% increase) was reached at 50 nM which corresponds to a 60% decrease in viability.

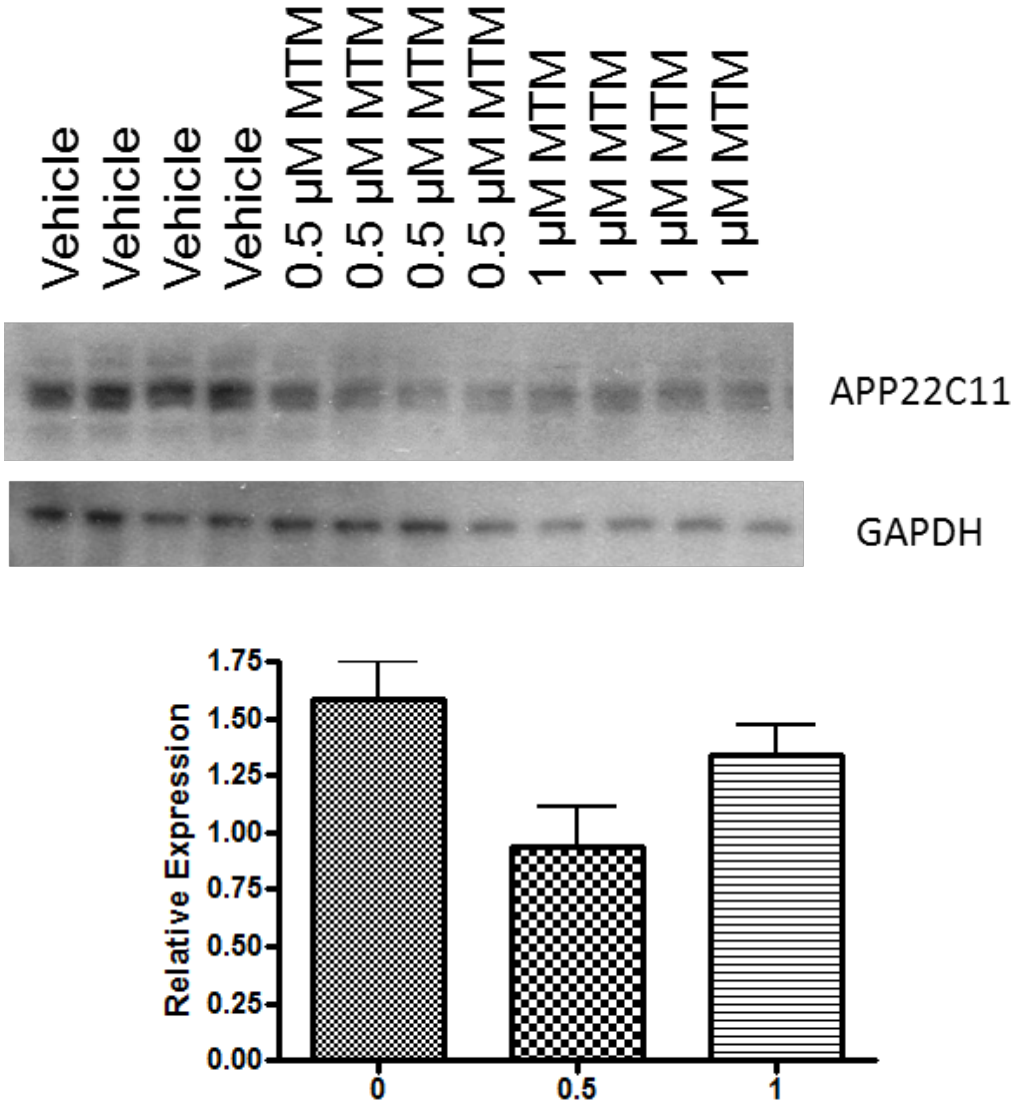


Figure 45. Effect of Mithramycin A on APP Expression in U373. U373 cultured in a 12-well plate; n=4; Vehicle (0.02% DMSO), 0.5, 1.0 µM Mithramycin A (MTM), 48 h treatment. GAPDH was used as a loading control and to normalize for densitometry. Relative APP levels were significantly decreased with MTM treatment, but not in a dose dependent manner.

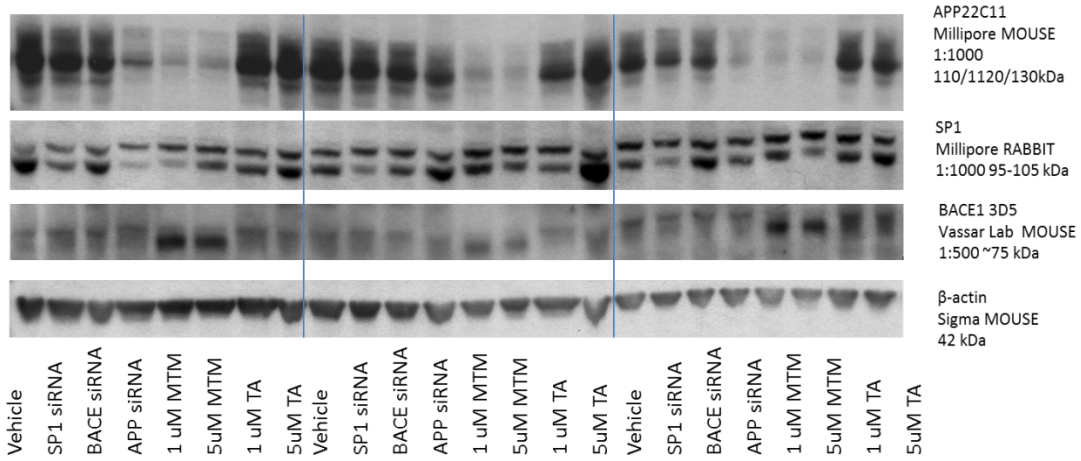


Figure 46. Effect of MTM and TA on APP, SP1, and BACE1 Protein Expression in U373. U373 were plated in a 24-well plate at 70% confluency. Cells were transfected with APP, BACE1, and SP1 siRNAs as controls (n=4). Other cells were treated with MTM or TA (1.0 or 5.0 μM). Beta-actin was used as the loading control in this instance. Both doses of MTM reduced APP levels in U373 and that TA causes no such change. Note that transfection with APP siRNA reduced APP expression as well, yet SP1 siRNA and BACE1 siRNA did not reduce their target proteins.

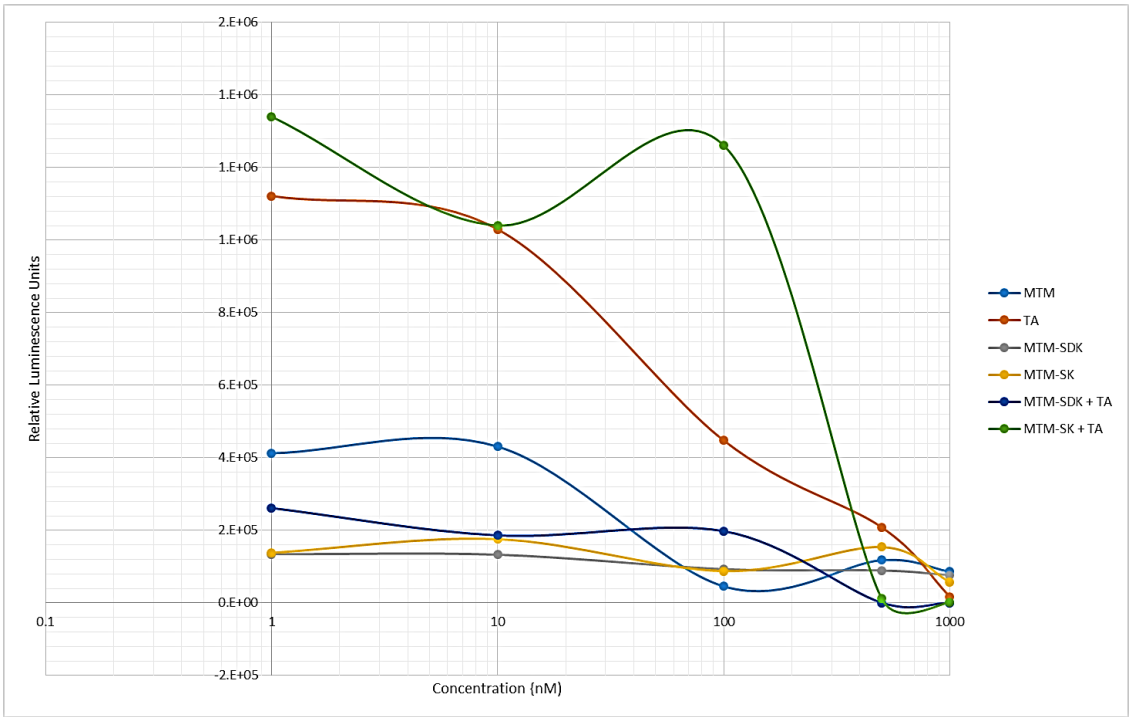


Figure 47. Effect of MTM, analogs, and TA on cell viability of human fetal neurons. Human fetal neurons were treated with MTM analogs MTM-SDK and MTM-SK alone and in combination with TA for 48 h. MTM analog MTM-SDK did not show a change in cell viability even at higher doses and in combination with TA. MTM-SK in combination with TA led to a significant decline in cell viability at the highest doses.

ii. Effect of transcription factor modulation on differentiated neurosphere cells

a. Effect of mithramycin A and tolfenamic acid on cell viability

Free-floating neurospheres were transferred from proliferation media to differentiation media in a 24-well plate format as previously described (Methods). At DIV10, cells were transfected with mock, SP1 siRNA, BACE1 siRNA, or APP siRNA (n=4). Cells in other wells were treated with two doses of either MTM or TA (n=4). Images were captured via microscopy and cell viability measured via CTG (Figure 49). SP1 inhibition by MTM, TA, or SP1 siRNA did not significantly affect cell viability of differentiated neurospheres as measured by CTG (Figure 49).

b. Effect of mithramycin A and tolfenamic acid on APP and SP1 levels

At DIV10, dNSPc were treated with vehicle, two doses of MTM or TA (1 μM or 5 μM) or combination treatment (1 μM) after 48 h (n=4). Western immunoblot analysis revealed a decrease in both APP and SP1 levels with MTM treatment (Figure 50). Combination treatment also led to a decrease in APP and SP1 levels in dNSPc, while TA treatment alone did not.

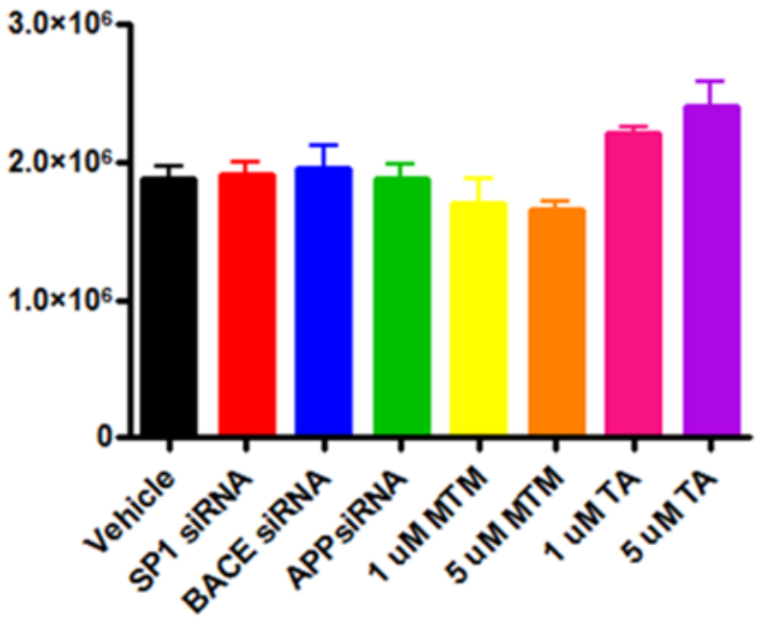


Figure 48. Effect of SP1 Modulation on cell morphology and viability of dNSPc. At DIV10, cells were transfected with mock, SP1 siRNA, BACE1 siRNA, or APP siRNA (n=4). Cells in other wells were treated with two doses of either MTM or TA (n=4). Images were captured via microscopy and cell viability measured via CTG after 48 h. SP1 inhibition by MTM, TA, or SP1 siRNA did not significantly affect cell viability of differentiated neurospheres as measured by CTG.

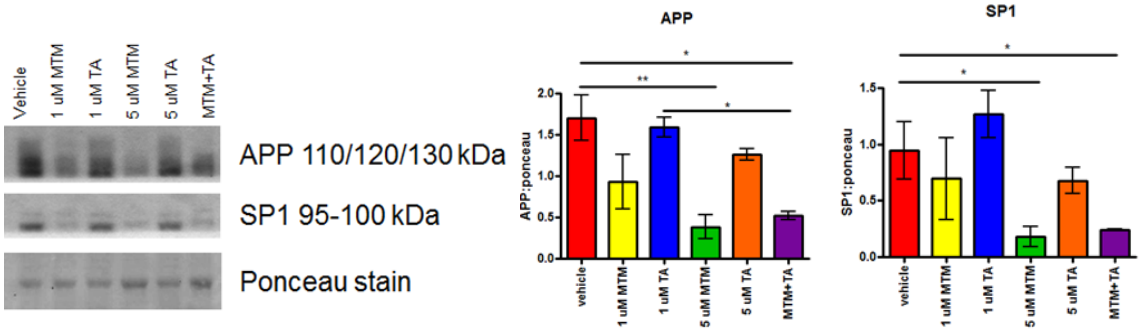


Figure 49. APP and SP1 levels measured by Western immunoblot in dNSPc. At DIV10, dNSPc were treated with vehicle, two doses of MTM or TA (1 μM or 5 μM) or combination treatment (1 μM) after 48 h (n=4). Ponceau stain was used to normalize protein loading. MTM, but not TA, treatment decreased APP and SP1 expression.

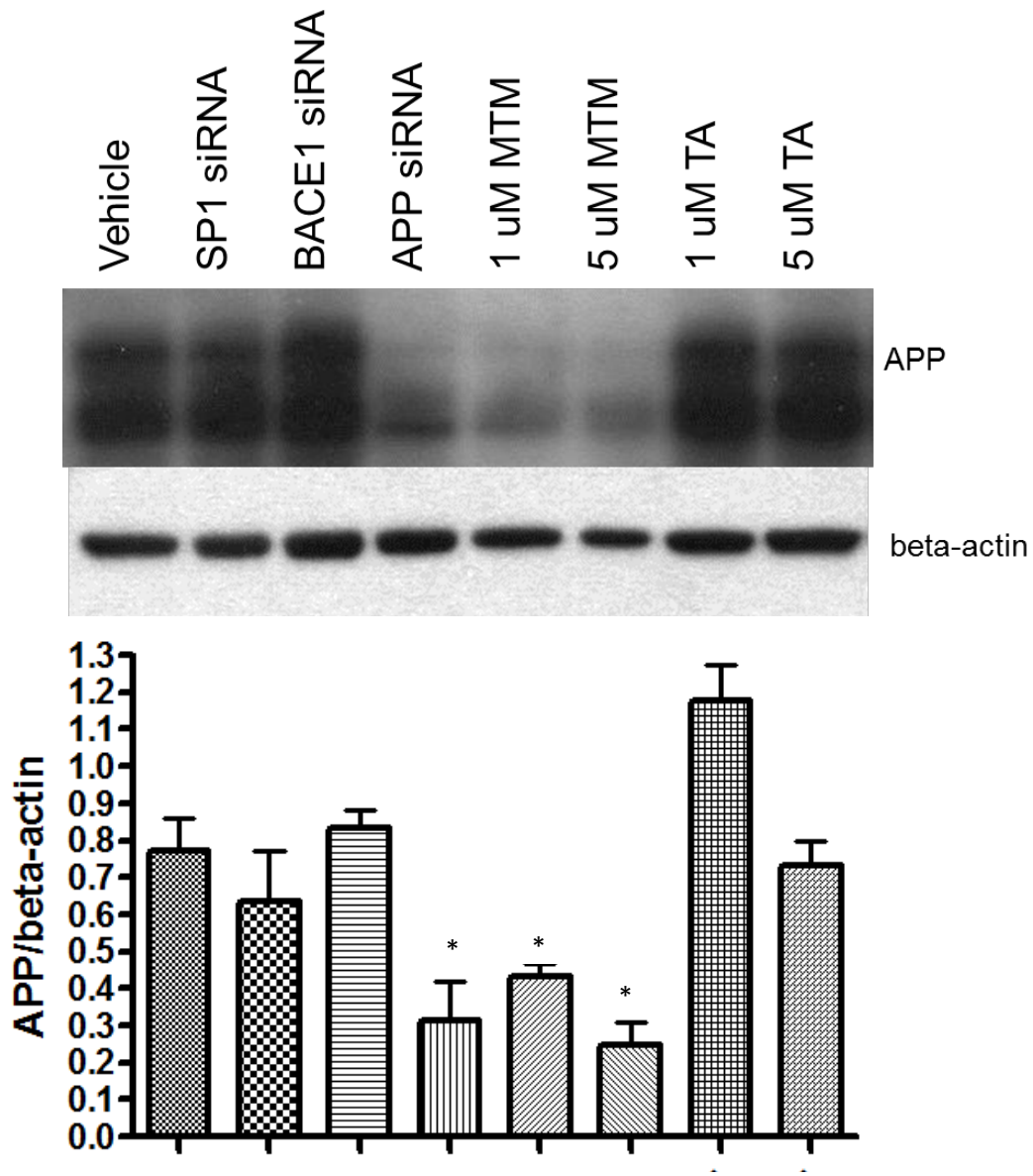


Figure 50. APP siRNA and MTM reduce APP levels in dNSPc. Western immunoblot and corresponding densitometry revealed a decrease in APP levels with treatment of the highest dose of MTM. Cells were treated in sets of 3 with error bars showing standard error of the mean.

c. Effect of mithramycin A and tolfenamic acid on amyloid-beta 40 levels

The sensitive sandwich ELISA measuring Aβ40 in conditioned media of cells treated for 48 h revealed both doses of MTM led to significant decreases in Aβ40 in differentiated neurospheres (Figure 51). By contrast, treatment with TA increased Aβ40 detected in dNSPc.

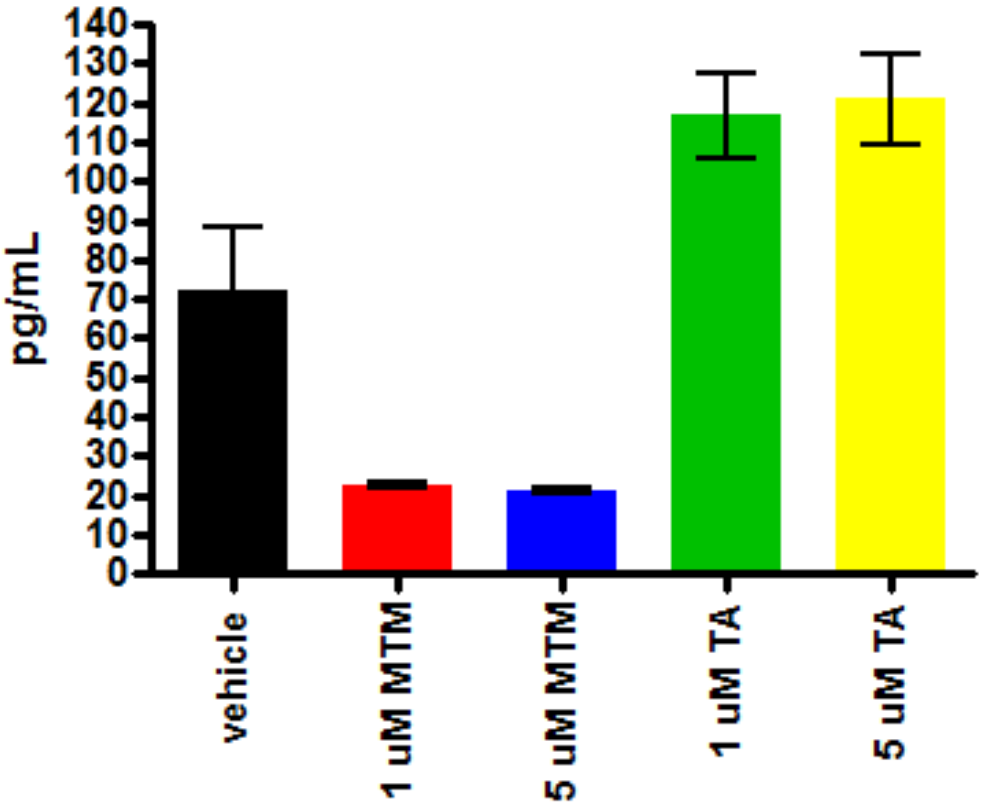


Figure 51. Specific Aβ40 sandwich ELISA. Sensitive sandwich ELISA measuring Aβ40 in conditioned media of cells treated for 48 h revealed both doses of MTM lead to significant decreases in Aβ40 in differentiated neurospheres.

iii. Effect of transcription factor modulation on differentiated neuroblastoma cells

DIV7 NBRA cells were transfected with mock or SP1 siRNA for 24 h in cells cultured on a 24-well plate. A total of 12 wells were transfected with SP1 siRNA. Drug treatments were added the following day. Vehicle (0.01% DMSO), MTM alone, TA alone, and combination MTM-TA treatment at 1 μM were added to cells. MTM was added to 4 wells transfected with SP1 siRNA and TA was added to another set of 4 wells previously transfected with SP1 siRNA (Figure 52). Conditioned media was collected at 48 h after drug treatment to assess cytotoxicity via LDH assay. Cells were harvested and lysed for cell viability reading via CTG assay.

a. Effect of mithramycin A or tolfenamic acid alone on cell viability and toxicity

MTM or TA treatment alone did not affect viability of NBRA cells. MTM treatment alone did significantly increase toxicity from vehicle by nearly 20%.

b. Effect of combination mithramycin A and tolfenamic acid treatment on cell viability and toxicity

Toxicity increased by nearly 40% in cells treated with MTM in combination with TA. Cell viability was reduced to undetectably low levels by CTG.

c. Effect of SP1 siRNA transfection on cell viability and toxicity

Knockdown of SP1 via siRNA alone did not change cell viability as compared to mock transfected cells. Cytotoxicity was not significantly different in cells transfected by mock compared to those transfected by SP1 siRNA.

d. Effect of SP1 siRNA transfection and tolfenamic acid treatment of cell viability

Knockdown of SP1 via siRNA in combination with TA leads to a nearly 70% LDH percent release by NBRA cells. Cell viability was reduced to undetectably low levels by CTG

e. Effect of SP1 siRNA transfection and mithramycin A treatment on cell viability

Treatment with MTM in combination with SP1 siRNA knockdown was well tolerated in human cells. Cell viability was not significantly different from mock and no increase in toxicity was detected by LDH measurement.

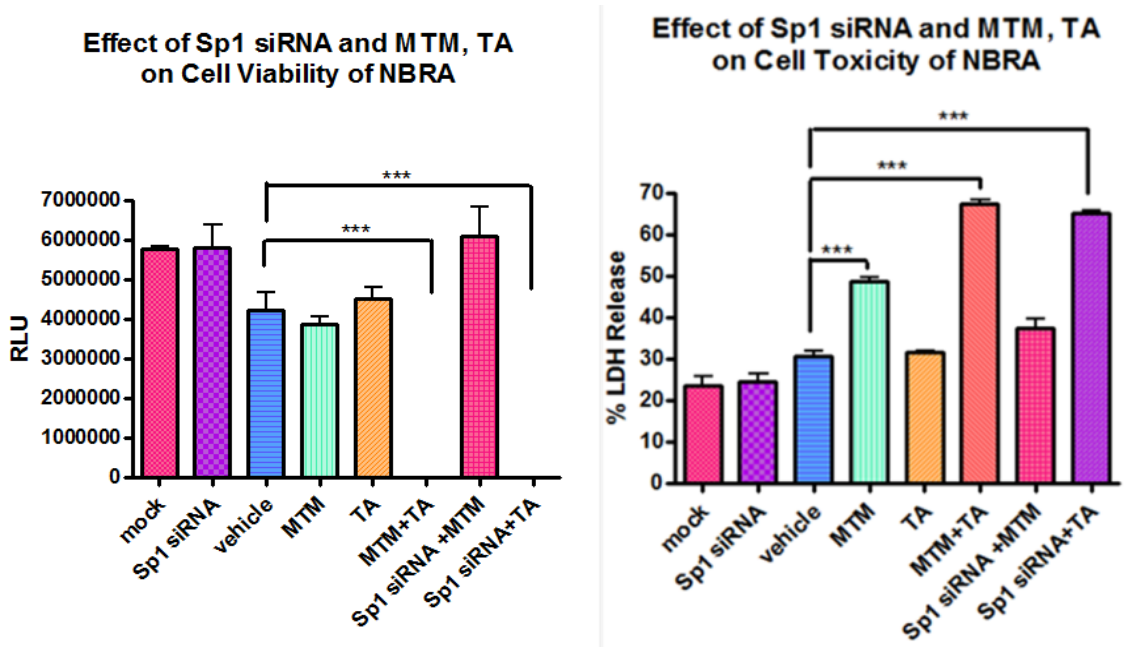


Figure 52. Effect of MTM and TA treatment on differentiated human neuroblastoma (NBRA) cells. MTM or TA treatment alone was non-toxic in NBRA (NBRA) cells as measured by CTG and LDH. Knockdown of SP1 via siRNA alone was also non-toxic. However, MTM in combination with TA was toxic and knockdown of SP1 via siRNA in combination with TA was toxic.

f. Effect of mithramycin A, its analogs, and tolfenamic acid on cell viability of NBRA

Nuclear extracts from NBRA treated with compounds showed variation in cell viability. MTM alone and MTM-SDK increased the cell viability (Figure 53).

g. Effect of mithramycin A, its analogs, and tolfenamic acid on SP1 levels by Western blot

Nuclear extracts from NBRA treated with compounds showed variation in SP1 levels (Figure 41). MTM treated neurons as well as those treated with combination MTM and TA showed a significant decrease in SP1 as compared to vehicle. Cells treated with analog MTM-SK and those treated with TA showed an increase in SP1 levels.

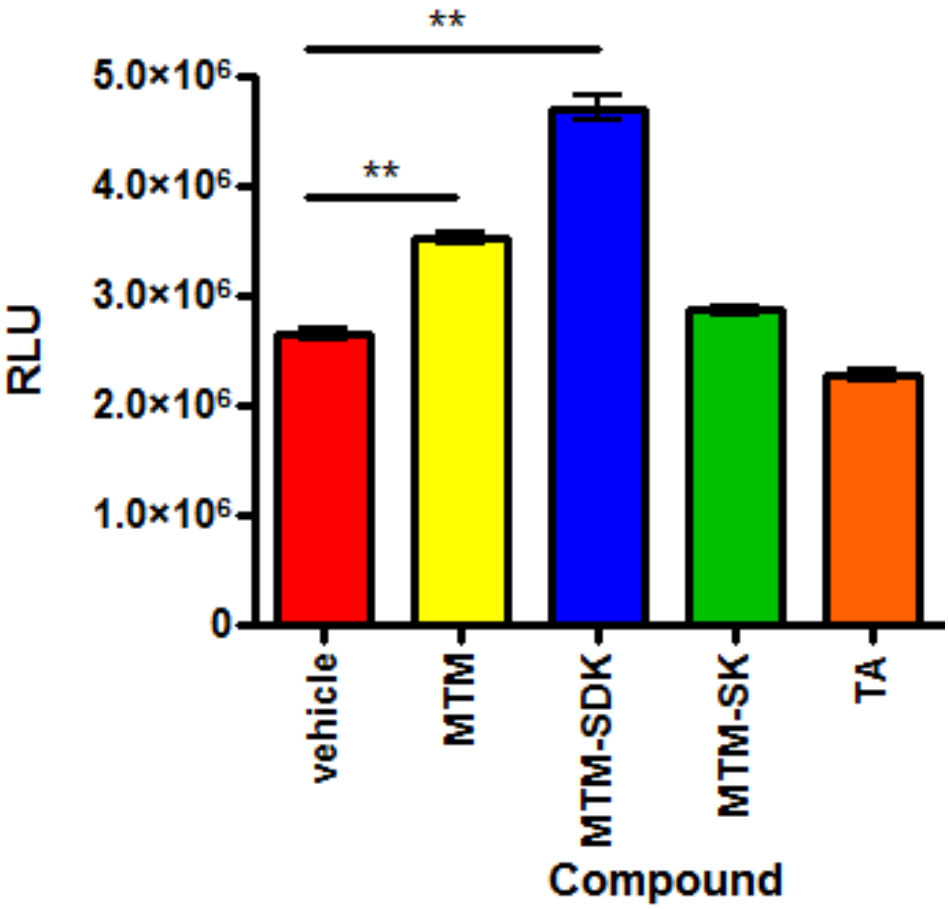


Figure 53. Effect of MTM, MTM analogs, and TA on cell viability of NBRA cells. Nuclear extracts from NBRA treated with compounds show variation in viability. Treatment with MTM and MTM-SDK increased cell viability as measured by CTG in NBRA. ** $p < 0.01$

h. Effect of mithramycin A, its analogs, and tolfenamic acid on neurite branch points

Time (h) was separated into three stages according to inflection of slope of treatment. Comparisons are of slopes within a stage. NBRA treated for 27 h were tracked via Incucyte ZOOM imaging. MTM and MTM-SK maintained number of neurite branch points per cell body area (Figure 54).

i. Effect of mithramycin A, its analogs, and tolfenamic acid on neurite length

Time (h) was separated into three stages according to inflection of slope of treatment. Comparisons are of slopes within a stage. NBRA treated for 27 h were tracked via Incucyte ZOOM imaging. MTM and MTM-SK maintained number of neurite lengths per cell body area (Figure 55).

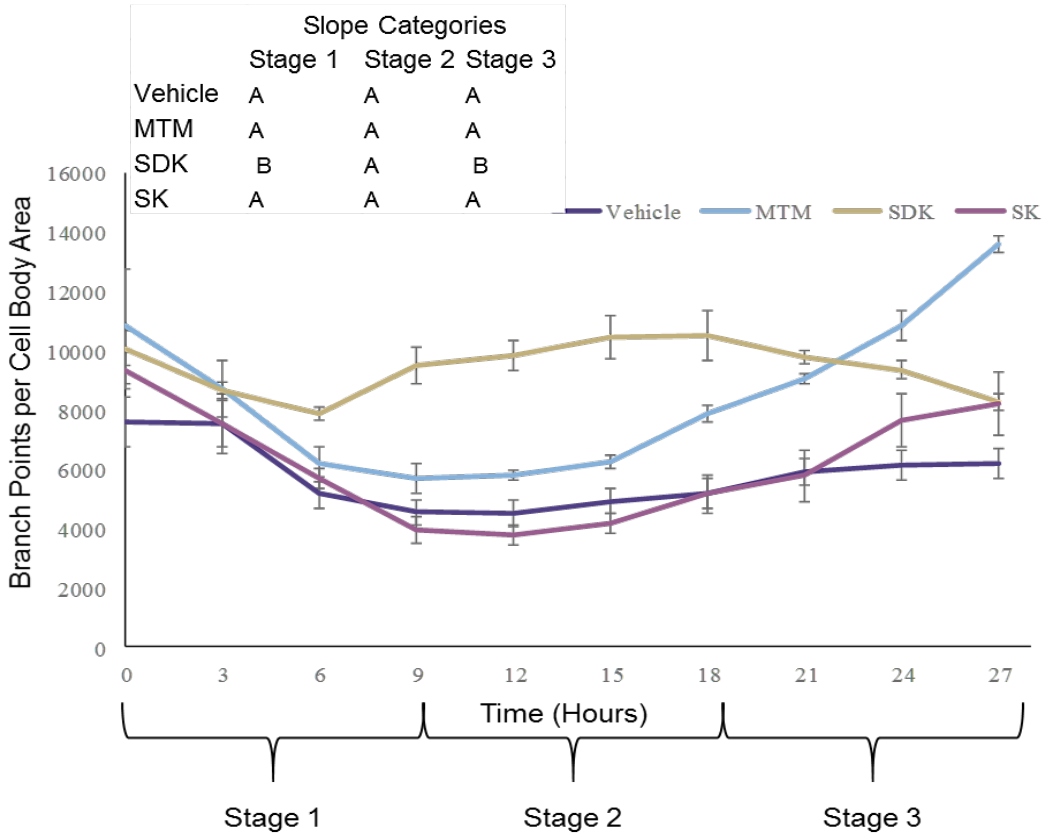


Figure 54. Changes in neurite branch points per cell body area in NBRA as measured by the Incucyte Zoom. Time (h) was separated into three stages according to inflection of slope of treatment. Comparisons are of slopes within a stage. NBRA treated for 27 h were tracked via Incucyte ZOOM imaging. MTM and MTM-SK maintained number of neurite branch points per cell body area.

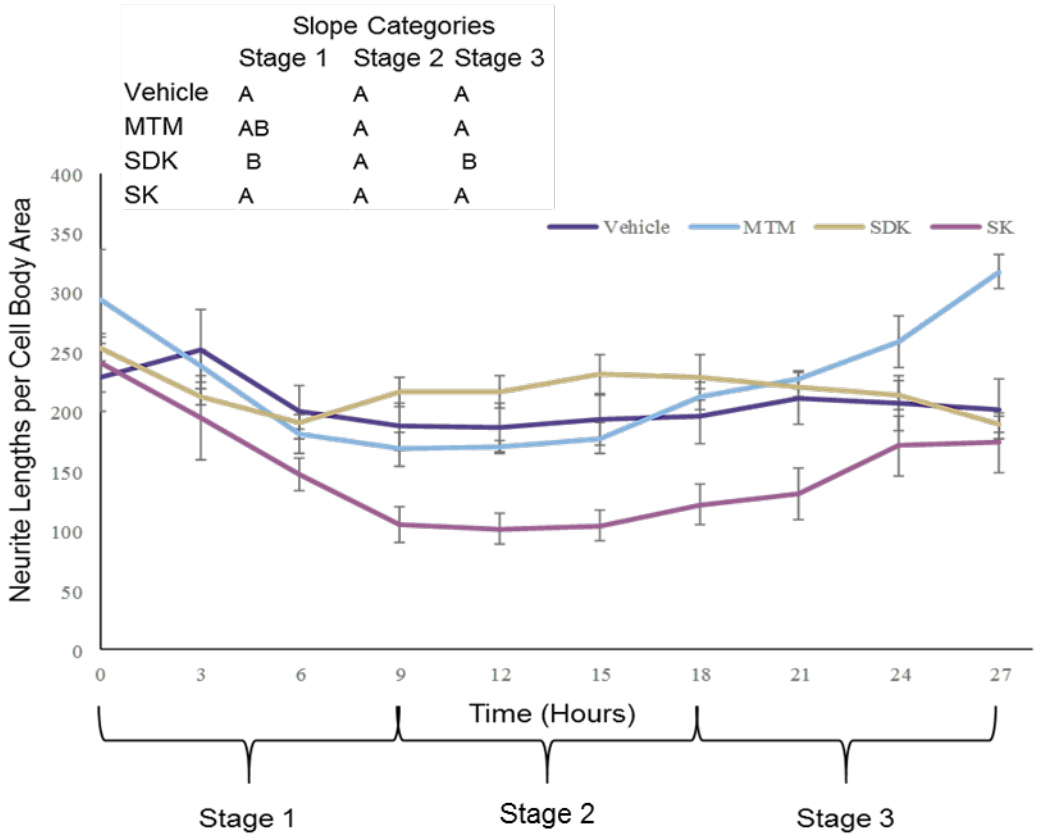
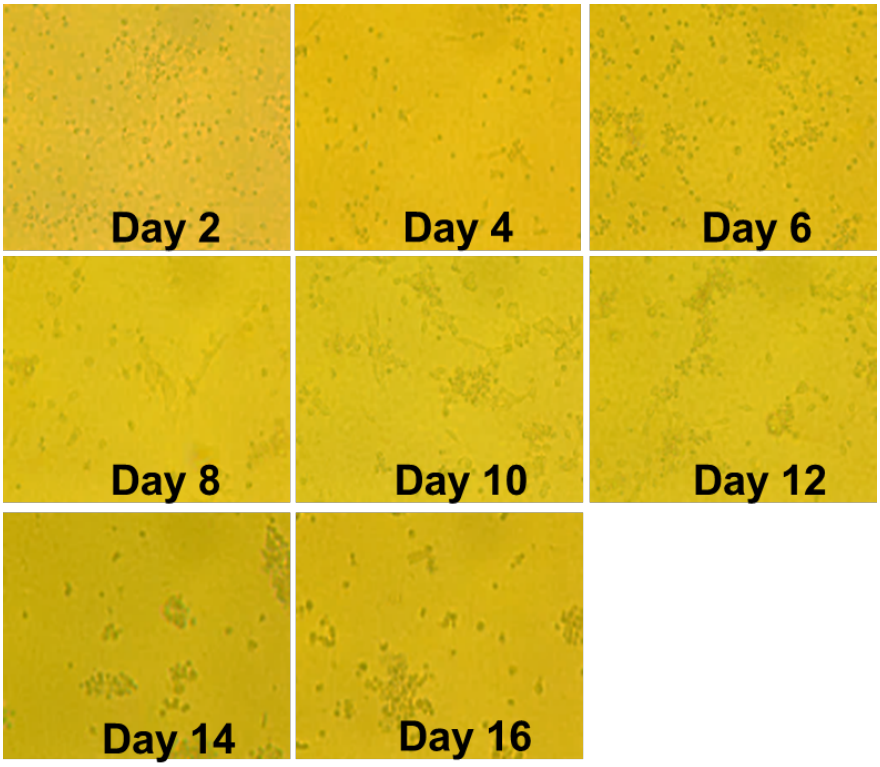


Figure 55. Changes in neurite lengths per cell body area in NBRA as measured by the Incucyte Zoom. Time (h) was separated into three stages according to inflection of slope of treatment. Comparisons are of slopes within a stage. NBRA treated for 27 h were tracked via Incucyte ZOOM imaging.

j. Induced pluripotent stem cell studies

Another valuable model for AD research includes induced fibroblasts from both control and AD subjects. This model allows the same advantages of cell culture while providing a tissue source directly from AD patients. Another advantage includes optimized, tailored media and reagents to support the cultures. Cells obtained from AXOL were cultured up to DIV 16 per manufacturer protocol (Figure 56) and assessed at different time points for changes in cell viability by CTG assay.

Control cells were cultured up to DIV10 and treated with vehicle or MTM for 48 h. Cells were harvested and lysed and assessed for cell viability via CTG (Figure 57). Treatment with MTM resulted in no significant change in cell viability of control iPSCs as compared to those treated with vehicle.



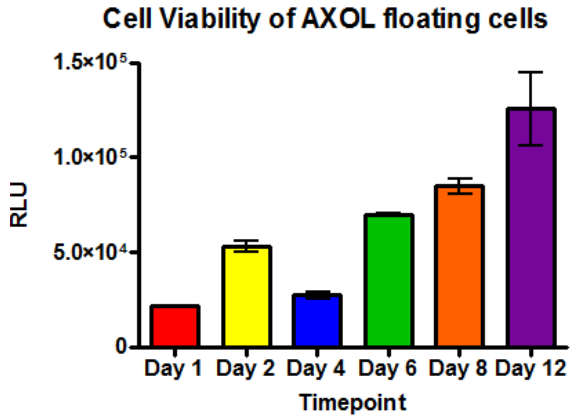


Figure 56. Human iPSC-derived neural stem cell studies. AXOL Human iPSC-Derived Neural Stem Cells - Alzheimer's Disease Patient (PSEN1 L286V mutation). Cells were cultured for up to 16 days with cells harvested at DIV2, 4, 6, 8, 10, 12, 14, and 16. Cell viability of each samples (n=3) was assessed via CTG assay up to DIV12.

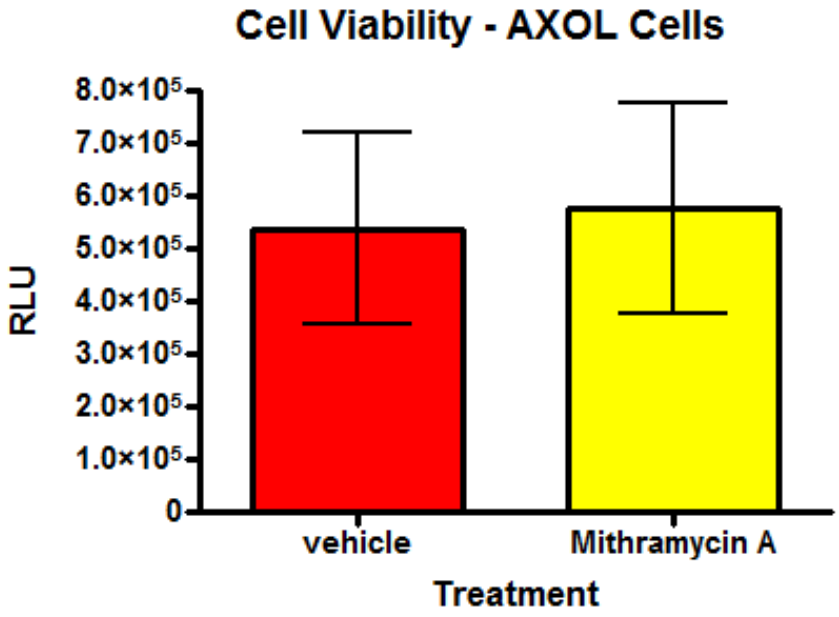


Figure 57. MTM in Control Patient iPSCs. Commercially available induced pluripotent stem cells were cultured for 10 days and treated with MTM or vehicle for 48 h. Treatment of iPSCs (control) with MTM (1 μM) showed no significant difference in viability versus vehicle.

iv. Understanding CDK5 modulation in human cells

a. Relative expression of FOXO1, FOXO3a and CDK5 proteins in human neuronal and glial cell lines and primary human brain cultures

A preliminary protein expression screen in cell lines and in lysates of primary brain cultures (Figure 58) revealed variable levels of forkhead box O 3a (Fox03a) protein in human neuronally-differentiated (HFN), glioblastoma, Jurkat cells and in primary human fetal brain cultures (HFB). The PC12 lysates in these experiments was later found to be degraded and data was not considered. Neuronal cell line SK-N-SH differentiated with retinoic acid (NBRA) shows a distinct band at the same molecular weight at the band seen in HFN. This band is also seen in the lysate of human T lymphocytes (jurkat). It is known that Foxo3a can regulate the differentiation, metabolism, survival, and cell cycle processes and may regulate these functions uniquely in differentiated neurons [181]. It has been previously shown that Foxo3a shows higher expression in the brain than does Foxo1 [182] . The data from the western blot in Figure 59was consistent with those findings as expression of FOXO1 protein levels were detected only in human glioblastoma (U373) and jurkat cells. Expression of CDK5 protein was detected only in human glioblastoma and jurkat cells as shown in the western blot analysis.

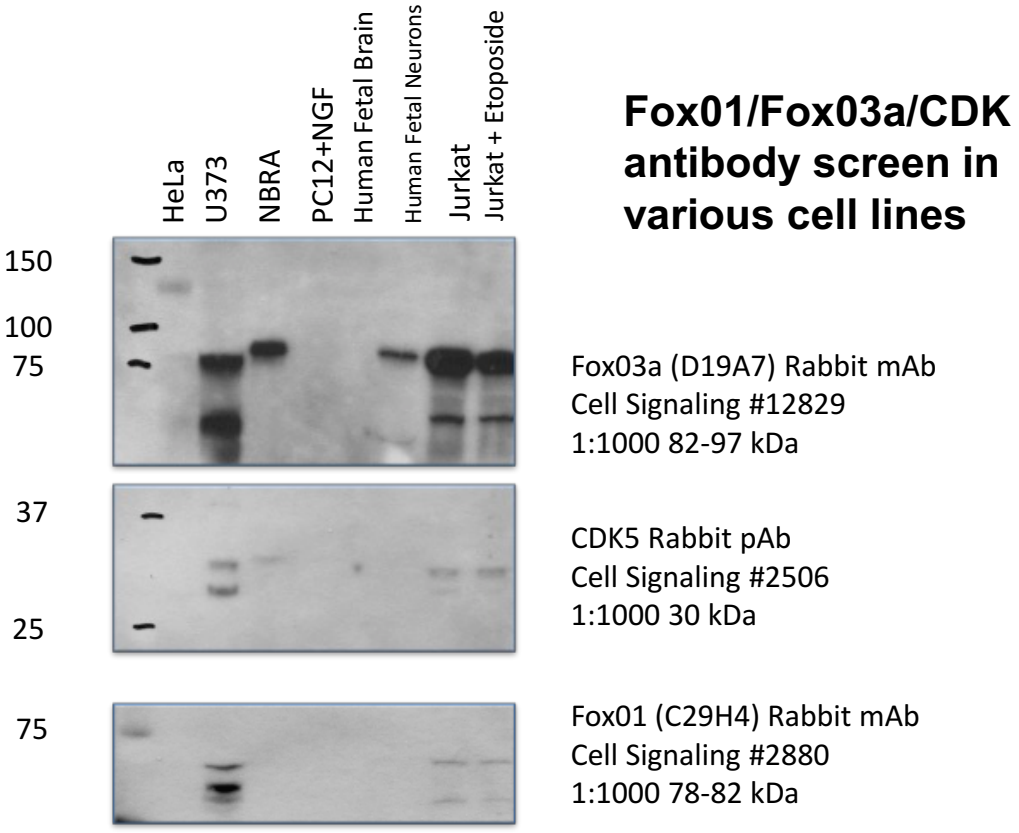


Figure 58. Foxo3a, Cdk5, and Foxo1 protein levels. Western blot analysis of Foxo3a, Cdk5, and Foxo1 in HeLa, U373, differentiated SK-N-SH, fetal brain, differentiated fetal neurons and Jurkat cells.

b. Relative expression of FOXO1, FOXO3a and CDK5 proteins between human fetal brain (HFBT) and human old brain tissue specimens (HOBT)

Western blot analysis of human brain samples of adult AD, and fetal origins revealed differences in expression of FOXO3a and CDK5 in these groups (Figure 59). There was a presence of FOXO3a protein in human old but not in fetal brain tissue. Interestingly, however, FOXO3a was detected in differentiated human fetal neurons that were derived from human fetal brain tissue. FOXO1 could not be detected in these samples and this data was not shown.

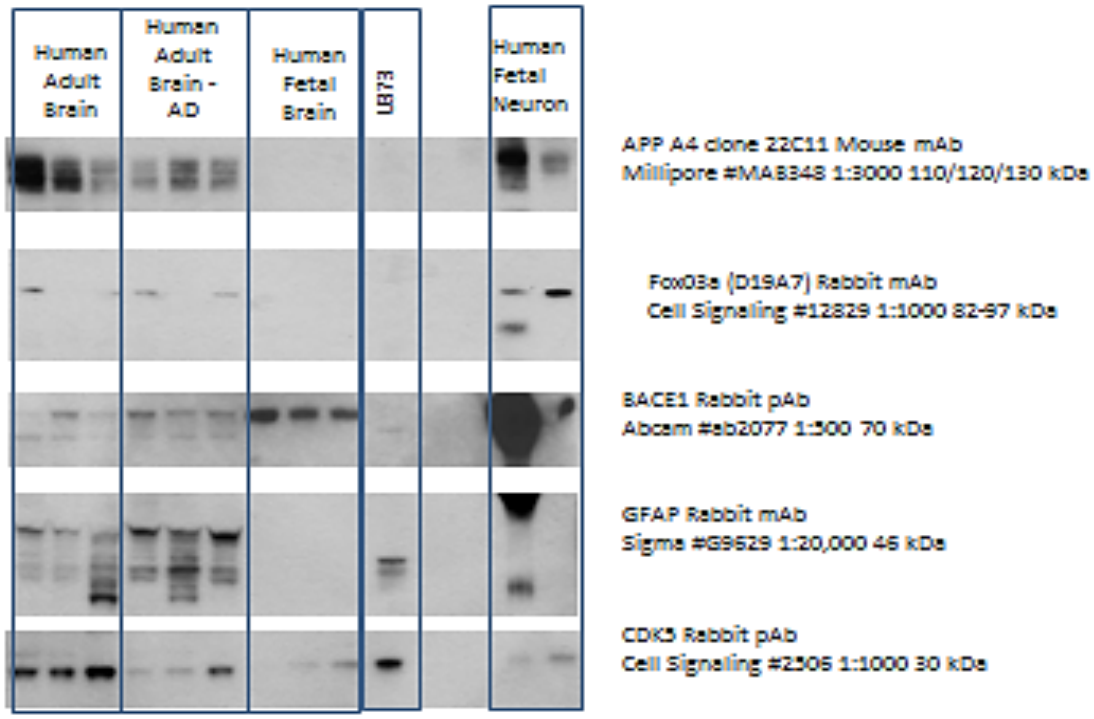


Figure 59. FOXO3a and CDK5 protein levels in various cell types.

Western blot analysis of FOXO3a, CDK5 in human adult brain samples from controls and Alzheimer's disease patients, U373 human glioblastoma cell line, and human fetal brain. The blot was re-probed with FOXO1 antibody, but no signal could be detected. APP, BACE1, and GFAP levels were also assessed in these samples.

c. Effects of Aβ1-42 challenge on the expression of FOXO1, FOXO3a and CDK5 proteins in human neuronal cultures

Differentiated human fetal neurons (DIV30) were treated with 1 μM Aβ1-42, 100 μM glutamate, or 100 μM H2O2 for 20 h (Figure 60). Human primary mixed brain cultures (HFB) were prepared and cultured as previously described (Ray et. al 2014). Briefly, cells were cultured in Neurobasal medium (Invitrogen) without phenol red supplemented with 1X B27, 50 mM GlutaMAX, 1x antibiotic cocktail, 5 ng/mL recombinant fibroblast growth factor 2 (bFGF) (Invitrogen), and 2 μL/mL Normocin (InVivoGen, San Diego, CA, USA). Cells were counted and seeded onto poly-D-lysine (Sigma-Aldrich) coated 24-well plates (Corning, Lowell, MA, USA) at 1.5 x 105 cells per well and maintained at 37°C in a 5% CO2 incubator. Half media changes were performed every fourth day of culture and morphology was monitored via IncucyteZOOM. Culture medium was removed from cells on day in vitro (DIV) 30 and replaced with Neurobasal medium with B27 minus antioxidant and Aβ1-42, H2O2, or glutamate. Cells were incubated at 37°C for 20 hours. Cells were harvested after 20 hours. Cells were rinsed with ice cold PBS, then lysed on plate with vigorous shaking using M-PER supplemented with 0.1% SDS and protease inhibitor (Roche). Protein concentration was estimated using the BCA Protein Assay (Pierce) per product protocol. Cell viability was measured by CTG levels.

d. Effect of roscovitine on cell morphology, cell viability of human fetal neurons

Roscovitine (ROSC) is a selective, competitive inhibitor of CDK5 [183]. We sought to assess the toxicity of this compound in human fetal neurons and the effect of treatment on levels of FOXO1 and FOXO3. Differentiated human fetal neurons (day in vitro 30) were treated with 1 μM Aβ1-42, 100 μM glutamate, or 100 μM H2O2 for 20 hours. Human primary mixed brain cultures (HFB) were prepared and cultured as previously described (Ray et. al 2014). Briefly, cells were cultured in Neurobasal medium (Invitrogen) without phenol red supplemented with 1X B27, 50 mM GlutaMAX, 1x antibiotic cocktail, 5 ng/mL recombinant fibroblast growth factor 2 (bFGF) (Invitrogen), and 2 μL/mL Normocin (InVivoGen, San Diego, CA, USA). Cells were counted and seeded onto poly-D-lysine (Sigma-Aldrich) coated 24-well plates (Corning, Lowell, MA, USA) at 1.5 x 105 cells per well and maintained at 37°C in a 5% CO2 incubator. Half media changes were performed every fourth day of culture and morphology was monitored via IncucyteZOOM. Culture medium was removed from cells on day in vitro (DIV) 31 and replaced with media with vehicle (2.5% DMSO), 0.1, 1.0, 10.0, 100.0, or 700.00 μM Roscovitine (Sigma) for 60 hours.

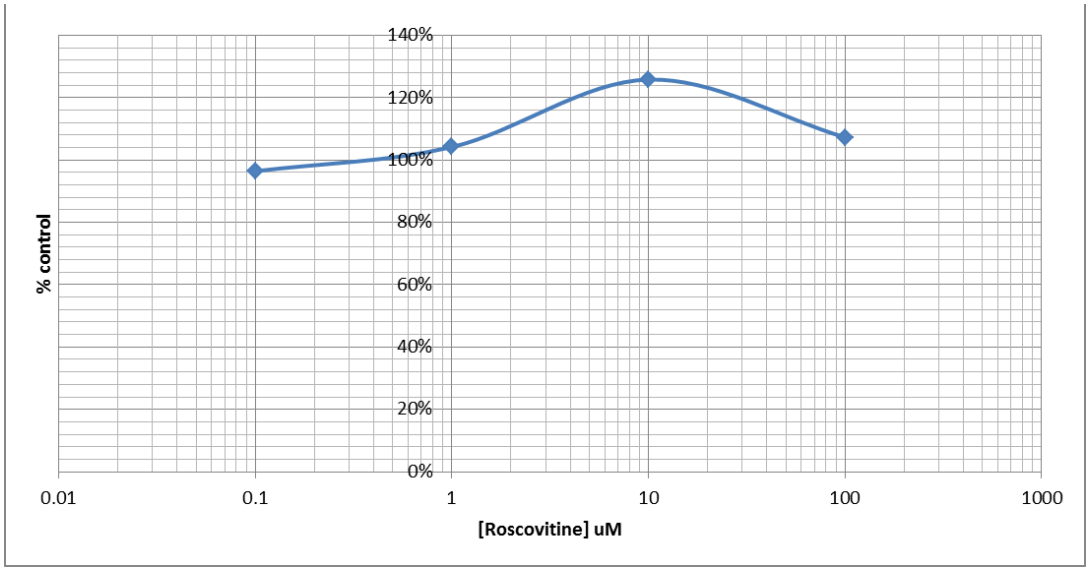


Figure 60. Effect of CDK5 inhibitor on cell viability of human fetal neurons. Treatment of HFN with increasing doses of roscovitine showed no significant change in cell viability as measured by CTG. % control is viability

C. Discussion

Of particular interest is the difference in the effect on viability and toxicity of MTM and TA when used in combination in a purely neuronal culture. This effect was not observed in the other cell culture systems. A compensatory mechanism involving other members of the SP family may account for the divergence in SP1 inhibition between MTM and TA when used separately and in combination in neurons. Use of MTM or the less toxic analog MTM-SDK to target SP1 in humans could potentially be a novel drug target in AD. Maintenance of synaptic health is a characteristic of interest in a disease involving massive neuronal and synaptic loss such as AD. MTM and MTM-SK maintain neurite length and neurite branch points, which could be key in preserving synaptic integrity in neurons early in the disease. Continued use of real-time live imaging of cells when treated with these compounds will provide increased insight into their effects on the synapse.

Preliminary data has shown tissue- and cell-specific expression of FOXO proteins and the effects of a CDK5 inhibiting drug on human cells. Future experiments should focus on inhibition of CDK5 to degrade FOXO3a as an approach to prevent or delay the neurodegeneration associated with AD. According to the CTG assay (Figure 60), doses of up to 100 μM of Roscovitine are well tolerated in human fetal neurons. Post-translational modifications control FOXO protein activity [184]. There could be modifications during the process of differentiation leading to activation of FOXO proteins. CDK5 expression in AD brains was decreased compared to controls. FOXO3a as a substrate of CDK5 may be differentially activated in AD. Levels appeared to be decreased in fetal

samples as compared to expression levels in adult samples. FOXO3 expression levels seemed to have an inverse relationship with BACE1 levels in these samples.

VII. Conclusions and future directions

A. Summary

Understanding how BACE1 can be regulated by a drug such as an antibiotic could lead to innovative therapies for patients suffering from AD. With the LEARn model as the mechanistic framework, discovering a way to regulate BACE1 activity in humans could serve as groundbreaking in later onset diseases such as AD. Use of drugs which target regulation of candidate genes at these earlier stages of disease will lead to improved bedside approach to patients presenting with early symptoms. Understanding these complex networks of TFs and investigating pharmacological strategies to modulate their activity can be a novel approach in AD. We believe that modest, yet specific reduction of BACE1 via SP1 modulation will provide effective and safe benefit to the brain without numerous adverse side effects in combination with existing therapies. Upon completion of this project, we have elucidated a possible mechanism by which SP1 regulates BACE1 activity and generation of Aβ in human cells. *A detailed understanding of the role SP1 plays in the amyloidogenic pathway in relation to the development of AD will have a positive impact by providing a new therapeutic target to slow the progression of this devastating disease.*

B. Other novel approaches to understanding amyloid pathway proteins

In addition to understanding the effect of TF modulation of Aβ pathway genes, further work should incorporate other novel approaches to targeting BACE1 and APP, including miRNAs and trafficking proteins.

i. Understanding the effect of miRNAs on the epigenome

Epigenetic regulation is often described as the alteration of gene expression without DNA sequence changes. These mechanisms are associated with histone modifications, DNA methylation, and microRNAs. MicroRNAs target transcripts, degrade them, and therefore control the amount of those particular proteins [185]. This directly influences the physiological state whether it be immediate or after a period of latency. It has been shown that miRNAs can be regulated by epigenetic means such as DNA methylation of their genes just as any other protein-coding gene [186, 187]. There are also miRNAs that can target DNA methyltransferases (DNMTs) [188]. Hypo- or hyper-methylation of the promoter regions of miRNAs can lead to these non-coding small RNAs functioning as tumor suppressors or other cancer prone phenotypes [189-191]. However, changes in mRNA translation due to miRNAs are not truly epigenomic because in their case, proteins are the active molecules involved [192]. Nevertheless, the close connection between the epigenome and miRNome means that epigenetically altered miRNAs can lead to pathogenesis that may function in a LEARned fashion [193]. The role of environment on the epigenome is a major focus on the LEARn model and its explanation of the progression of late onset neurodegenerative diseases such as Alzheimer's disease (AD) and other psychiatric disorders [167, 168, 194]. How we interact with the environment may indeed affect how miRNAs are expressed and regulated over time. Lifestyle, diet, chemical exposure, and trauma are all environmental influences that may lead to epigenetic "hits" that affect the genome in a latent

manner. We know that childhood abuse and adversity can lead to methylation changes [195, 196]. Propensity for addiction can be highly influenced by stress and other environmental factors [197]. MiRNAs may modulate the cocaine response in individuals for which stress has had an epigenetic effect in early-life. It has been shown that there is a relationship between miRNA expression and reactions to fear and other emotional stimuli [198]. There are miRNAs which are induced in anxiety which regulate neurotransmitter pathways and can lead to dysregulation [199]. Some of these miRNAs respond to acute stress and may even contribute to depressive disorders [200, 201]. Studies have also revealed that overexpression of miRNAs is seen in the brains of AD mice, suggesting a potential link between these non-coding small RNAs and behavior [202]. It is quite possible that changes in DNA methylation caused by these stress or anxiety states are passed along through the germline. There are several examples of the LEARn model functioning in a transgenerational manner [80]. It is not yet clear how epigenetic changes in miRNA expression can be inherited; it has recently been shown that elevated levels of certain miRNAs may be a marker for behavioral disorders across generations [203] .

The case can be made that miRNA function in the same axis of the epigenetic process. Because of their unique biogenesis and mechanism of action via mRNA targeting, they cannot be completely categorized as epigenomic in nature. However, because of their unique interaction role in regulation of epigenetic processes and their ability to be regulated my epigenetic means, this

relationship merits examinations as putative approach to diseases which manifest in a latent manner.

ii. Understanding the role of APP and BACE1 trafficking

The regulation of APP and BACE1 activity is important in the goal of reducing amyloid-beta. However, understanding how APP and BACE1 physically interact at the cellular level is key to elucidating alternative strategies for changing their prevalence in AD.

BACE1 is first produced in the endoplasmic reticulum (ER) as a larger precursor, pro-BACE1. Pro-BACE1 is subsequently modified by post-translational glycosylation where the prodomain is removed allowing this protein to cross the Golgi apparatus. BACE1 is then transported to the cell surface where BACE1 functions in the regulation of cellular signaling [204]. BACE1 is activated through phosphorylation at serine 498 by casein kinase I, and the dephosphorylation of this residue leads to endocytosis of BACE1 from the plasma membrane. As a result, BACE1 subsequently accumulates in early endosomes [205]. Previous studies demonstrate that BACE1 recycles between endosomes and the surface of the cell [206, 207]. In order for BACE1 to remain in the Golgi where APP processing occurs, a single transmembrane domain is required [208].

Physical proximity as well as duration plays an important role in the ability of enzymes and their substrates to interact with one another, and both factors are essential to the processing of APP via BACE1. Therefore, understanding the cellular conditions such as protein-protein interactions and cellular signaling

which modulate this proximity and subsequent convergence of APP and BACE1 may provide an important mechanistic key to the regulation of Aβ production. Likewise, manipulation of trafficking pathways that decrease the interaction could provide a method to inhibit the production of Aβ and possibly provide a cellular target to prevent AD pathogenesis or treat the symptoms of AD.

APP is cleaved by BACE1 to form $sAPP_{\beta,}$ the secreted ectodomain and CTF99, the C-terminal fragment. Aβ is then liberated from APP is known as regulated intramembrane proteolysis (RIP) [209]. Aβ is comprised of a cluster of heterogenous peptides of 39-43 amino acids [17]. While the most prevalent peptide is $A\beta_{40}$, the 42-amino acid peptide ($A\beta_{42}$) has been identified as the critical isoform associated with AD. Aβ is typically released in its soluble form into the interstitial fluid (ISF) in the brain [21]. However, Aβ aggregates as both soluble oligomers and insoluble plaques in AD brain [210]. Pro-BACE1 in the ER also displays enzymatic activity that can cleave APP [211]. Consistent with this phenomenon, a minority of $A\beta_{42}$ is generated in the ER, while a greater percentage of $A\beta_{42}$ is observed in the trans-Golgi network [212, 213]. Therefore, differences in APP processing by BACE1 in the ER leading to Aβ aggregation may provide a key factor in the pathogenesis of AD.

BACE1 is a transmembrane aspartyl protease with an acidic pH-optimum of 4.5 [28]. BACE1 is first localized to the Golgi, and then migrates to the trans-Golgi network and finally to endosomes. Previous studies indicate that BACE1 transport may proceed due to several mechanisms. For instance, one study found that BACE1 may use an endosomal retrograde transport pathway [214].

Other studies indicate that BACE1 is transported from early endosomes to lysosomes [215], and may be recycled from endosomes to the plasma membrane [216].

The retromer complex is a heteropentameric complex that can be separated into two subcomplexes [217]. The trimer component consists of three active sites that mediate cargo selection including Vps35, Vps29, and Vps26. The dimer component consists of Vps5 and Vps17. In mammals, these components are known as members of the sorting nexin family and called SNX1 or SNX2 and SNX5 or SNX6. Vps35 is the main component of the trimer. Studies demonstrate that reductions in Vps35 can lead to retromer malfunction [218].

The trimer is responsible for trafficking Vps10 from the vacuole to the *trans*-Golgi. If excessive levels of APP or BACE1 are present, the trimer is unable to function properly leading to a buildup of Aβ in the endosome. The selective targeting of this cellular mechanism may provide a method that can be used to modulate protein trafficking of APP and BACE1, and ultimately Aβ levels. In a recent study, the Mecozzi et al. propose that pharmacological chaperones can be utilized to manipulate retromer complex stability, thereby affecting how its cargo (i.e., APP and BACE) are trafficked throughout the cell. BACE1 and APP convergence are critical for the processing of APP. The manner in which APP converges with BACE-1 after neuronal activity indicates that neurons have developed a distinctive tactic to limit the proximity of APP and BACE-1. Perhaps the dysregulation of this mechanism may contribute to increased Aβ production and the pathology of AD.

iii. Unraveling the LEARn Model

The link between TFs and AD-relevant processes has been shown. It however, remains unclear the molecular mechanism of drugs that may target these TFs and how this can be manipulated at the cellular level. Accumulation of Aβ peptide in the brain remains a key focus of AD pathology. We postulate that non-familial AD is a late onset disease during which early-life exposure (i.e. nutrition, experiences, etc.) can lead to epigenetic changes over a period of latency that at some later time are triggered to alter phenotypic state. We describe this hypothesis as the latent early life regulation (LEARn) model. Such an early-life exposure that may contribute to this sporadic disease could be the environmental toxin lead (Pb). Pb exposure has been part of recent alarming headlines as related to toxic levels in the water of residents of Flint, Michigan in the United States. Pb has long been associated with cognitive decline in humans [219]. In rodents, early exposure to Pb resulted in poor performance on cognitive tests in aged animals. [220]. Exposure to Pb in monkeys during development leads to an increase in expression of amyloid-precursor protein (*APP*) and that of its rate-limiting cleaving enzyme, *BACE1* [221]. The TF known to regulate both of these genes, SP1, was also increased in subjects exposed to Pb during development.

In order to test the LEARn hypothesis, experiments using a mouse model with human APP, BACE1, PSEN, and MAPT would be ideal for use. Performing standard behavioral tests of these animals would be necessary to establish a baseline of epigenetic status. In a select group of mice, trauma, neglect,

exposure to a toxin, or other insult would be introduced at a very young age (perhaps during nursing). Varying periods of time of the exposure and different period of time for length of exposure would have to be carefully chosen. Behavior would need to be observed over time and compared to the control group later in life. Again, methylation status and histone acetylation activity would be measured as well as a battery of behavioral tests. If in fact mice exposed to an early life insult show changes in methylation that correspond to a decrease in cognitive function, increase in BACE1 activity, increased plaque and tangle production, along with changes in APP, MAPT, and PSEN, this would be strong evidence to support the hypothesis that LEARn is a mechanism for the development of AD.

In future studies, we seek to understand the specific regulation of AD-relevant genes by SP1 and the ability of compounds to modulate SP1 in cultures with or without previous exposure to Pb. Primary human brain cultures and induced pluripotent stem cells from controls and AD subjects will be used to test the effects of drugs previously shown to decrease Aβ; mithramycin A (MTM) and its analogs (MTM-SDK, MTM-SK) at a later time point to observe changes in SP1 and other TFs, as well as Aβ from neurons exposed to Pb and those without Pb treatment. We wish to understand if Pb is functioning to trigger a period of epigenetic latency, and if so, how markers such as histone deacetylase (HDAC), DNA methyltransferase activity, and TF levels are affected over time. Also of interest is whether MTM and its analogs can alter these changes when used to treat cells after a period of latency. We expect that early Pb exposure leads to

changes in DNA methylation or histone acetylation in neurons that can be attenuated via TF-modulating drugs.AD is likely caused by several risk alleles across many different loci. These genes also likely affect several pathways involved in the clearance or production of Aβ. Neuroimaging consists fMRI, PET-FDG, and other brain imaging techniques to gain structural and functional information on biomarkers used for AD classification and early diagnosis. Genome-wide association studies will involve searching for SNPs located in genes of TFs that may be important according to AD imaging biomarkers. This work will help to elucidate a possible mechanism for TF regulation of AD-related genes via well-characterized compounds in primary culture models as well as by candidate SNP searches in the AD Neuroimaging (ADNI) database. The impact of understanding how environmental influences such as Pb exposure affect the epigenome of neurons and discovering how compounds can potentially reverse these influences is tremendous. At a time when a massive number of US citizens have been highly exposed to toxic levels of Pb, it is very important to understand the mechanism by which early life changes caused by these tragic exposures may be reversed with available compounds such at MTM and its analogs.

C. Conclusions

The development of new cell culture model systems in neuroscience is imperative. Use of human models in AD study is invaluable as rodent studies are not translating into successful clinical trial studies in human subjects. The human fetal neuron culture and the human neurosphere culture both are mixed brain models that recapitulate the cellular populations of the CNS. With this work, human dNSPCs, iPSCs, and NBRA cells have been successfully cultured and tested with novel drugs. Testing compounds such as MTM, its analogs, TA, and roscovitine in a human system allows a more direct connection to effects that may be observed in patients in more advanced stages of study.

As use of MTM and TA in humans is extensive and has been well documented, we propose repurposing these drugs at nontoxic doses for use in AD to reduce amyloid-beta. Specifically, MTM analogs should be more closely examined for their effects on neurobiology and amyloid-beta levels in these primary human cultures as they have been here in cell lines. As MTM-SDK was shown to decrease DNMT activity, this may enhance or decrease the ability of SP1 to bind to target DNA. Future studies should focus on how this lesser methyltransferase activity directly affects SP1 and other TF binding relevant to AD.

Broadening our view of TF targeting to include epigenetic modifications as well as miRNAs could be novel approaches to neurodegenerative disease therapies. Furthermore, compounds that can alter APP and BACE1 trafficking to decrease the proximity of the β-secretase to its substrate may be useful for

decreasing amyloid burden. Lastly, targeting multiple transcription repressors and activators such as the FOXO proteins can help us alter AD phenotypes as well. These unique and novel approaches, perhaps if used in combination will prove more promising than any of them may be alone.

Removing amyloid once people have established dementia may be the source of failure of prior approaches to AD therapy. AD may in face be a continuum that begins well before any detectable changes in cognition. Drugging TFs that regulate amyloid pathway genes still assumes the amyloid hypothesis is correct, however if tolerated could be given much earlier on the AD spectrum when early biomarkers become available. Although TFs have commonly been viewed as “un-druggable,” their function as the regulators of gene expression make them absolutely necessary in the understanding of the mechanism of novel approaches to diseases such as AD that have alluded other solutions for so long. It is imperative that these networks continue to be studied and described so that treatment strategies can be made available for such a devastating disease.

REFERENCES

1. Hebert, L.E., et al., *Alzheimer disease in the US population: prevalence estimates using the 2000 census.* Arch Neurol, 2003. **60**(8): p. 1119-22.
2. Alzheimer, A., et al., *An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde".* Clin Anat, 1995. **8**(6): p. 429-31.
3. Hebert, L.E., et al., *Alzheimer disease in the United States (2010-2050) estimated using the 2010 census.* Neurology, 2013. **80**(19): p. 1778-83.
4. Murphy, S.L., J. Xu, and K.D. Kochanek, *Deaths: final data for 2010.* Natl Vital Stat Rep, 2013. **61**(4): p. 1-117.
5. Ganguli, M. and E.G. Rodriguez, *Reporting of dementia on death certificates: a community study.* J Am Geriatr Soc, 1999. **47**(7): p. 842-9.
6. Tinetti, M.E., et al., *Contribution of individual diseases to death in older adults with multiple diseases.* J Am Geriatr Soc, 2012. **60**(8): p. 1448-56.
7. Ganguli, M., et al., *Alzheimer disease and mortality: a 15-year epidemiological study.* Arch Neurol, 2005. **62**(5): p. 779-84.
8. Waring, S.C., et al., *Survival among patients with dementia from a large multi-ethnic population.* Alzheimer Dis Assoc Disord, 2005. **19**(4): p. 178-83.
9. Brookmeyer, R., et al., *Survival following a diagnosis of Alzheimer disease.* Arch Neurol, 2002. **59**(11): p. 1764-7.
10. Larson, E.B., et al., *Survival after initial diagnosis of Alzheimer disease.* Ann Intern Med, 2004. **140**(7): p. 501-9.

11. Arrighi, H.M., et al., *Lethality of Alzheimer disease and its impact on nursing home placement.* Alzheimer Dis Assoc Disord, 2010. **24**(1): p. 90-5.
12. Alzheimer's, A., *2016 Alzheimer's disease facts and figures.* Alzheimers Dement, 2016. **12**(4): p. 459-509.
13. Coleman, P.D. and P.J. Yao, *Synaptic slaughter in Alzheimer's disease.* Neurobiol Aging, 2003. **24**(8): p. 1023-7.
14. Bailey, J.A., et al., *Rivastigmine lowers Abeta and increases sAPPalpha levels, which parallel elevated synaptic markers and metabolic activity in degenerating primary rat neurons.* PLoS One, 2011. **6**(7): p. e21954.
15. Blennow, K., M.J. de Leon, and H. Zetterberg, *Alzheimer's disease.* Lancet, 2006. **368**(9533): p. 387-403.
16. Gatz, M., et al., *Role of genes and environments for explaining Alzheimer disease.* Arch Gen Psychiatry, 2006. **63**(2): p. 168-74.
17. Glenner, G.G. and C.W. Wong, *Alzheimer's disease and Down's syndrome: sharing of a unique cerebrovascular amyloid fibril protein.* Biochem Biophys Res Commun, 1984. **122**(3): p. 1131-5.
18. Hardy, J.A. and G.A. Higgins, *Alzheimer's disease: the amyloid cascade hypothesis.* Science, 1992. **256**(5054): p. 184-5.
19. Price, D.L., et al., *Alzheimer's disease: genetic studies and transgenic models.* Annu Rev Genet, 1998. **32**: p. 461-93.
20. Scheuner, D., et al., *Secreted amyloid beta-protein similar to that in the senile plaques of Alzheimer's disease is increased in vivo by the presenilin*

1 and 2 and APP mutations linked to familial Alzheimer's disease. Nat Med, 1996. **2**(8): p. 864-70.

21. Vassar, R., *BACE1: the beta-secretase enzyme in Alzheimer's disease.* J Mol Neurosci, 2004. **23**(1-2): p. 105-14.
22. Tanzi, R.E. and L. Bertram, *Twenty years of the Alzheimer's disease amyloid hypothesis: a genetic perspective.* Cell, 2005. **120**(4): p. 545-55.
23. Hardy, J., *Amyloid double trouble.* Nat Genet, 2006. **38**(1): p. 11-2.
24. Dong, H., et al., *Spatial relationship between synapse loss and beta-amyloid deposition in Tg2576 mice.* J Comp Neurol, 2007. **500**(2): p. 311-21.
25. Meyer-Luehmann, M., et al., *A reporter of local dendritic translocation shows plaque- related loss of neural system function in APP-transgenic mice.* J Neurosci, 2009. **29**(40): p. 12636-40.
26. Buckner, R.L., et al., *Molecular, structural, and functional characterization of Alzheimer's disease: evidence for a relationship between default activity, amyloid, and memory.* J Neurosci, 2005. **25**(34): p. 7709-17.
27. Selkoe, D.J. and D. Schenk, *Alzheimer's disease: molecular understanding predicts amyloid-based therapeutics.* Annu Rev Pharmacol Toxicol, 2003. **43**: p. 545-84.
28. Rossner, S., et al., *Transcriptional and translational regulation of BACE1 expression--implications for Alzheimer's disease.* Prog Neurobiol, 2006. **79**(2): p. 95-111.

29. Haass, C., *Take five--BACE and the gamma-secretase quartet conduct Alzheimer's amyloid beta-peptide generation.* EMBO J, 2004. **23**(3): p. 483-8.

30. Cai, H., et al., *BACE1 is the major beta-secretase for generation of Abeta peptides by neurons.* Nat Neurosci, 2001. **4**(3): p. 233-4.

31. Luo, Y., et al., *Mice deficient in BACE1, the Alzheimer's beta-secretase, have normal phenotype and abolished beta-amyloid generation.* Nat Neurosci, 2001. **4**(3): p. 231-2.

32. Vassar, R., et al., *Beta-secretase cleavage of Alzheimer's amyloid precursor protein by the transmembrane aspartic protease BACE.* Science, 1999. **286**(5440): p. 735-41.

33. Yan, R., et al., *Membrane-anchored aspartyl protease with Alzheimer's disease beta-secretase activity.* Nature, 1999. **402**(6761): p. 533-7.

34. Bigl, M., et al., *Expression of beta-secretase mRNA in transgenic Tg2576 mouse brain with Alzheimer plaque pathology.* Neurosci Lett, 2000. **292**(2): p. 107-10.

35. Marcinkiewicz, M. and N.G. Seidah, *Coordinated expression of beta-amyloid precursor protein and the putative beta-secretase BACE and alpha-secretase ADAM10 in mouse and human brain.* J Neurochem, 2000. **75**(5): p. 2133-43.

36. von Arnim, C.A., et al., *The low density lipoprotein receptor-related protein (LRP) is a novel beta-secretase (BACE1) substrate.* J Biol Chem, 2005. **280**(18): p. 17777-85.

37. Li, Q. and T.C. Sudhof, *Cleavage of amyloid-beta precursor protein and amyloid-beta precursor-like protein by BACE 1.* J Biol Chem, 2004. **279**(11): p. 10542-50.

38. Pastorino, L., et al., *BACE (beta-secretase) modulates the processing of APLP2 in vivo.* Mol Cell Neurosci, 2004. **25**(4): p. 642-9.

39. Kitazume, S., et al., *Alzheimer's beta-secretase, beta-site amyloid precursor protein-cleaving enzyme, is responsible for cleavage secretion of a Golgi-resident sialyltransferase.* Proc Natl Acad Sci U S A, 2001. **98**(24): p. 13554-9.

40. Lichtenthaler, S.F., et al., *The cell adhesion protein P-selectin glycoprotein ligand-1 is a substrate for the aspartyl protease BACE1.* J Biol Chem, 2003. **278**(49): p. 48713-9.

41. Hu, X., et al., *Genetic deletion of BACE1 in mice affects remyelination of sciatic nerves.* FASEB J, 2008. **22**(8): p. 2970-80.

42. Willem, M., et al., *Control of peripheral nerve myelination by the beta-secretase BACE1.* Science, 2006. **314**(5799): p. 664-6.

43. Kim, D.Y., et al., *Presenilin/gamma-secretase-mediated cleavage of the voltage-gated sodium channel beta2-subunit regulates cell adhesion and migration.* J Biol Chem, 2005. **280**(24): p. 23251-61.

44. Wong, H.K., et al., *beta Subunits of voltage-gated sodium channels are novel substrates of beta-site amyloid precursor protein-cleaving enzyme (BACE1) and gamma-secretase.* J Biol Chem, 2005. **280**(24): p. 23009-17.

45. Fukumoto, H., et al., *Beta-secretase activity increases with aging in human, monkey, and mouse brain.* Am J Pathol, 2004. **164**(2): p. 719-25.

46. Fukumoto, H., et al., *Beta-secretase protein and activity are increased in the neocortex in Alzheimer disease.* Arch Neurol, 2002. **59**(9): p. 1381-9.

47. Yang, L.B., et al., *Elevated beta-secretase expression and enzymatic activity detected in sporadic Alzheimer disease.* Nat Med, 2003. **9**(1): p. 3-4.

48. Li, R., et al., *Amyloid beta peptide load is correlated with increased beta-secretase activity in sporadic Alzheimer's disease patients.* Proc Natl Acad Sci U S A, 2004. **101**(10): p. 3632-7.

49. Tyler, S.J., et al., *alpha- and beta-secretase: profound changes in Alzheimer's disease.* Biochem Biophys Res Commun, 2002. **299**(3): p. 373-6.

50. Hu, X., et al., *BACE1 regulates hippocampal astrogenesis via the Jagged1-Notch pathway.* Cell Rep, 2013. **4**(1): p. 40-9.

51. Hu, X., et al., *Bace1 modulates myelination in the central and peripheral nervous system.* Nat Neurosci, 2006. **9**(12): p. 1520-5.

52. Sankaranarayanan, S., et al., *In vivo beta-secretase 1 inhibition leads to brain Abeta lowering and increased alpha-secretase processing of amyloid precursor protein without effect on neuregulin-1.* J Pharmacol Exp Ther, 2008. **324**(3): p. 957-69.

53. Citron, M., et al., *Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein production.* Nature, 1992. **360**(6405): p. 672-4.

54. Gruninger-Leitch, F., et al., *Substrate and inhibitor profile of BACE (beta-secretase) and comparison with other mammalian aspartic proteases.* J Biol Chem, 2002. **277**(7): p. 4687-93.

55. Ge, Y.W., et al., *Functional characterization of the 5' flanking region of the BACE gene: identification of a 91 bp fragment involved in basal level of BACE promoter expression.* FASEB J, 2004. **18**(9): p. 1037-9.

56. Lahiri, D.K., et al., *Taking down the unindicted co-conspirators of amyloid beta-peptide-mediated neuronal death: shared gene regulation of BACE1 and APP genes interacting with CREB, Fe65 and YY1 transcription factors.* Curr Alzheimer Res, 2006. **3**(5): p. 475-83.

57. Sambamurti, K., et al., *Gene structure and organization of the human beta-secretase (BACE) promoter.* FASEB J, 2004. **18**(9): p. 1034-6.

58. Cruts, M., J. Theuns, and C. Van Broeckhoven, *Locus-specific mutation databases for neurodegenerative brain diseases.* Hum Mutat, 2012. **33**(9): p. 1340-4.

59. Zheng, H., et al., *beta-Amyloid precursor protein-deficient mice show reactive gliosis and decreased locomotor activity.* Cell, 1995. **81**(4): p. 525-31.

60. Lopes, J.P., C.R. Oliveira, and P. Agostinho, *Neurodegeneration in an Abeta-induced model of Alzheimer's disease: the role of Cdk5.* Aging Cell, 2010. **9**(1): p. 64-77.

61. Shah, K. and D.K. Lahiri, *Cdk5 activity in the brain - multiple paths of regulation.* J Cell Sci, 2014. **127**(Pt 11): p. 2391-400.

62. Shah, K. and D.K. Lahiri, *A Tale of the Good and Bad: Remodeling of the Microtubule Network in the Brain by Cdk5.* Mol Neurobiol, 2017. **54**(3): p. 2255-2268.

63. Monaco, E.A., 3rd, *Recent evidence regarding a role for Cdk5 dysregulation in Alzheimer's disease.* Curr Alzheimer Res, 2004. **1**(1): p. 33-8.

64. Zhou, J., et al., *The roles of Cdk5-mediated subcellular localization of FOXO1 in neuronal death.* J Neurosci, 2015. **35**(6): p. 2624-35.

65. Shi, C., et al., *Cdk5-Foxo3 axis: initially neuroprotective, eventually neurodegenerative in Alzheimer's disease models.* J Cell Sci, 2016. **129**(9): p. 1815-1830.

66. Sambamurti, K., et al., *Targets for AD treatment: conflicting messages from gamma-secretase inhibitors.* J Neurochem, 2011. **117**(3): p. 359-74.

67. Bartus, R.T., et al., *The cholinergic hypothesis of geriatric memory dysfunction.* Science, 1982. **217**(4558): p. 408-14.

68. Davis, K.L., et al., *Cholinergic markers in elderly patients with early signs of Alzheimer disease.* JAMA, 1999. **281**(15): p. 1401-6.

69. Nygaard, H.B., *Current and emerging therapies for Alzheimer's disease.* Clin Ther, 2013. **35**(10): p. 1480-9.

70. Long, J.M. and D.K. Lahiri, *MicroRNA-101 downregulates Alzheimer's amyloid-beta precursor protein levels in human cell cultures and is differentially expressed.* Biochem Biophys Res Commun, 2011. **404**(4): p. 889-95.

71. Czech, M.P., M. Aouadi, and G.J. Tesz, *RNAi-based therapeutic strategies for metabolic disease.* Nat Rev Endocrinol, 2011. **7**(8): p. 473-84.

72. Becker, R.E. and N.H. Greig, *Why so few drugs for Alzheimer's disease? Are methods failing drugs?* Curr Alzheimer Res, 2010. **7**(7): p. 642-51.

73. Doody, R.S., et al., *A phase 3 trial of semagacestat for treatment of Alzheimer's disease.* N Engl J Med, 2013. **369**(4): p. 341-50.

74. Coric, V., et al., *Safety and tolerability of the gamma-secretase inhibitor avagacestat in a phase 2 study of mild to moderate Alzheimer disease.* Arch Neurol, 2012. **69**(11): p. 1430-40.

75. Lahiri, D.K., et al., *Lessons from a BACE1 inhibitor trial: Off-site but not off base.* Alzheimers Dement, 2014.

76. Sacks, C.A., J. Avorn, and A.S. Kesselheim, *The Failure of Solanezumab - How the FDA Saved Taxpayers Billions.* N Engl J Med, 2017. **376**(18): p. 1706-1708.

77. The Lancet, N., *Solanezumab: too late in mild Alzheimer's disease?* Lancet Neurol, 2017. **16**(2): p. 97.

78. Christensen, M.A., et al., *Transcriptional regulation of BACE1, the beta-amyloid precursor protein beta-secretase, by Sp1.* Mol Cell Biol, 2004. **24**(2): p. 865-74.

79. Basha, M.R., et al., *The fetal basis of amyloidogenesis: exposure to lead and latent overexpression of amyloid precursor protein and beta-amyloid in the aging brain.* J Neurosci, 2005. **25**(4): p. 823-9.

80. Lahiri, D.K., et al., *Transgenerational latent early-life associated regulation unites environment and genetics across generations.* Epigenomics, 2016. **8**(3): p. 373-87.

81. Lombo, F., et al., *The aureolic acid family of antitumor compounds: structure, mode of action, biosynthesis, and novel derivatives.* Appl Microbiol Biotechnol, 2006. **73**(1): p. 1-14.

82. Barcelo, F., et al., *Entropically-driven binding of mithramycin in the minor groove of C/G-rich DNA sequences.* Nucleic Acids Res, 2007. **35**(7): p. 2215-26.

83. Sleiman, S.F., et al., *Mithramycin is a gene-selective Sp1 inhibitor that identifies a biological intersection between cancer and neurodegeneration.* J Neurosci, 2011. **31**(18): p. 6858-70.

84. Choi, E.S., et al., *Mithramycin A induces apoptosis by regulating the mTOR/Mcl-1/tBid pathway in androgen-independent prostate cancer cells.* J Clin Biochem Nutr, 2013. **53**(2): p. 89-93.

85. Albertini, V., et al., *Novel GC-rich DNA-binding compound produced by a genetically engineered mutant of the mithramycin producer Streptomyces*

argillaceus exhibits improved transcriptional repressor activity: implications for cancer therapy. Nucleic Acids Res, 2006. **34**(6): p. 1721-34.

86. Abdelfattah, M.S. and J. Rohr, *Premithramycinone G, an early shunt product of the mithramycin biosynthetic pathway accumulated upon inactivation of oxygenase MtmOII.* Angew Chem Int Ed Engl, 2006. **45**(34): p. 5685-9.

87. Ryan, W.G., *Mithramycin for Paget's disease of bone.* N Engl J Med, 1970. **283**(21): p. 1171.

88. Ryan, W.G., T.B. Schwartz, and G. Northrop, *Experiences in the treatment of Paget's disease of bone with mithramycin.* JAMA, 1970. **213**(7): p. 1153-7.

89. Seznec, J., B. Silkenstedt, and U. Naumann, *Therapeutic effects of the Sp1 inhibitor mithramycin A in glioblastoma.* J Neurooncol, 2011. **101**(3): p. 365-77.

90. Gao, Y., et al., *Combining betulinic acid and mithramycin a effectively suppresses pancreatic cancer by inhibiting proliferation, invasion, and angiogenesis.* Cancer Res, 2011. **71**(15): p. 5182-93.

91. Grohar, P.J., et al., *Identification of an inhibitor of the EWS-FLI1 oncogenic transcription factor by high-throughput screening.* J Natl Cancer Inst, 2011. **103**(12): p. 962-78.

92. Bianchi, N., et al., *Targeting of the Sp1 binding sites of HIV-1 long terminal repeat with chromomycin. Disruption of nuclear factor.DNA complexes*

and inhibition of in vitro transcription. Biochem Pharmacol, 1996. **52**(10): p. 1489-98.

93. Chatterjee, S., et al., *Sequence-selective DNA binding drugs mithramycin A and chromomycin A3 are potent inhibitors of neuronal apoptosis induced by oxidative stress and DNA damage in cortical neurons.* Ann Neurol, 2001. **49**(3): p. 345-54.

94. Ferrante, R.J., et al., *Chemotherapy for the brain: the antitumor antibiotic mithramycin prolongs survival in a mouse model of Huntington's disease.* J Neurosci, 2004. **24**(46): p. 10335-42.

95. Voisine, C., et al., *Identification of potential therapeutic drugs for huntington's disease using Caenorhabditis elegans.* PLoS One, 2007. **2**(6): p. e504.

96. Hagiwara, H., M. Iyo, and K. Hashimoto, *Mithramycin protects against dopaminergic neurotoxicity in the mouse brain after administration of methamphetamine.* Brain Res, 2009. **1301**: p. 189-96.

97. Sen, C.K., et al., *Molecular basis of vitamin E action. Tocotrienol potently inhibits glutamate-induced pp60(c-Src) kinase activation and death of HT4 neuronal cells.* J Biol Chem, 2000. **275**(17): p. 13049-55.

98. Khanna, S., et al., *Glutamate-induced c-Src activation in neuronal cells.* Methods Enzymol, 2002. **352**: p. 191-8.

99. Khanna, S., et al., *Characterization of the potent neuroprotective properties of the natural vitamin E alpha-tocotrienol.* J Neurochem, 2006. **98**(5): p. 1474-86.

100. Stanciu, M., et al., *Persistent activation of ERK contributes to glutamate-induced oxidative toxicity in a neuronal cell line and primary cortical neuron cultures.* J Biol Chem, 2000. **275**(16): p. 12200-6.

101. Chin, P.C., et al., *The c-Raf inhibitor GW5074 provides neuroprotection in vitro and in an animal model of neurodegeneration through a MEK-ERK and Akt-independent mechanism.* J Neurochem, 2004. **90**(3): p. 595-608.

102. Baldus, C.D., et al., *Acute myeloid leukemia with complex karyotypes and abnormal chromosome 21: Amplification discloses overexpression of APP, ETS2, and ERG genes.* Proc Natl Acad Sci U S A, 2004. **101**(11): p. 3915-20.

103. Zhu, X., et al., *Activation of oncogenic pathways in degenerating neurons in Alzheimer disease.* Int J Dev Neurosci, 2000. **18**(4-5): p. 433-7.

104. Previll, L.A., et al., *Increased expression of p130 in Alzheimer disease.* Neurochem Res, 2007. **32**(4-5): p. 639-44.

105. Kennedy, B.J., *Metabolic and toxic effects of mithramycin during tumor therapy.* Am J Med, 1970. **49**(4): p. 494-503.

106. Brown, J.H. and B.J. Kennedy, *Mithramycin in the Treatment of Disseminated Testicular Neoplasms.* N Engl J Med, 1965. **272**: p. 111-8.

107. Koller, C.A. and D.M. Miller, *Preliminary observations on the therapy of the myeloid blast phase of chronic granulocytic leukemia with plicamycin and hydroxyurea.* N Engl J Med, 1986. **315**(23).

108. Weidenbach, S., et al., *Dimerization and DNA recognition rules of mithramycin and its analogues.* J Inorg Biochem, 2016. **156**: p. 40-7.

109. Previdi, S., et al., *Inhibition of Sp1-dependent transcription and antitumor activity of the new aureolic acid analogues mithramycin SDK and SK in human ovarian cancer xenografts.* Gynecol Oncol, 2010. **118**(2): p. 182-8.

110. Sastry, M. and D.J. Patel, *Solution structure of the mithramycin dimer-DNA complex.* Biochemistry, 1993. **32**(26): p. 6588-604.

111. Sastry, M., R. Fiala, and D.J. Patel, *Solution structure of mithramycin dimers bound to partially overlapping sites on DNA.* J Mol Biol, 1995. **251**(5): p. 674-89.

112. Malek, A., et al., *Modulation of the activity of Sp transcription factors by mithramycin analogues as a new strategy for treatment of metastatic prostate cancer.* PLoS One, 2012. **7**(4): p. e35130.

113. Scott, D., et al., *Semi-synthetic mithramycin SA derivatives with improved anticancer activity.* Chem Biol Drug Des, 2013. **81**(5): p. 615-24.

114. Scott, D., J. Rohr, and Y. Bae, *Nanoparticulate formulations of mithramycin analogs for enhanced cytotoxicity.* Int J Nanomedicine, 2011. **6**: p. 2757-67.

115. Remsing, L.L., et al., *Ketopremithramycins and ketomithramycins, four new aureolic acid-type compounds obtained upon inactivation of two genes involved in the biosynthesis of the deoxysugar moieties of the antitumor drug mithramycin by Streptomyces argillaceus, reveal novel insights into post-PKS tailoring steps of the mithramycin biosynthetic pathway.* J Am Chem Soc, 2002. **124**(8): p. 1606-14.

116. Corell, T., *Pharmacology of tolfenamic acid.* Pharmacol Toxicol, 1994. **75 Suppl 2**: p. 14-21.

117. Abdelrahim, M., et al., *Tolfenamic acid and pancreatic cancer growth, angiogenesis, and Sp protein degradation.* J Natl Cancer Inst, 2006. **98**(12): p. 855-68.

118. Adwan, L., G.M. Subaiea, and N.H. Zawia, *Tolfenamic acid downregulates BACE1 and protects against lead-induced upregulation of Alzheimer's disease related biomarkers.* Neuropharmacology, 2014. **79**: p. 596-602.

119. Adwan, L.I., et al., *Tolfenamic acid interrupts the de novo synthesis of the beta-amyloid precursor protein and lowers amyloid beta via a transcriptional pathway.* Curr Alzheimer Res, 2011. **8**(4): p. 385-92.

120. Subaiea, G.M., et al., *Short-term treatment with tolfenamic acid improves cognitive functions in Alzheimer's disease mice.* Neurobiol Aging, 2013. **34**(10): p. 2421-30.

121. Adwan, L., et al., *Tolfenamic acid reduces tau and CDK5 levels: implications for dementia and tauopathies.* J Neurochem, 2014.

122. Lee, S.H., et al., *Activating transcription factor 2 (ATF2) controls tolfenamic acid-induced ATF3 expression via MAP kinase pathways.* Oncogene, 2010. **29**(37): p. 5182-92.

123. Shao, H.J., et al., *Tolfenamic Acid Suppresses Inflammatory Stimuli-Mediated Activation of NF-kappaB Signaling.* Biomol Ther (Seoul), 2015. **23**(1): p. 39-44.

124. Sankpal, U.T., et al., *Cellular and organismal toxicity of the anti-cancer small molecule, tolfenamic acid: a pre-clinical evaluation.* Cell Physiol Biochem, 2013. **32**(3): p. 675-86.

125. Medina, M., *Recent developments in tau-based therapeutics for neurodegenerative diseases.* Recent Pat CNS Drug Discov, 2011. **6**(1): p. 20-30.

126. Subaiea, G.M., et al., *Reduction of amyloid-beta deposition and attenuation of memory deficits by tolfenamic acid.* J Alzheimers Dis, 2015. **43**(2): p. 425-33.

127. Hakkarainen, H., et al., *Tolfenamic acid and caffeine: a useful combination in migraine.* Cephalalgia, 1982. **2**(4): p. 173-7.

128. Sutphin, R.M., et al., *Anti-leukemic response of a NSAID, tolfenamic acid.* Target Oncol, 2014. **9**(2): p. 135-44.

129. Marin, M., et al., *Transcription factor Sp1 is essential for early embryonic development but dispensable for cell growth and differentiation.* Cell, 1997. **89**(4): p. 619-28.

130. Eskerod, O., *Gastrointestinal tolerance studies on tolfenamic acid in humans and animals.* Pharmacol Toxicol, 1994. **75 Suppl 2**: p. 44-8.

131. Cruz, J.C., et al., *p25/cyclin-dependent kinase 5 induces production and intraneuronal accumulation of amyloid beta in vivo.* J Neurosci, 2006. **26**(41): p. 10536-41.

132. Wen, Y., et al., *Transcriptional regulation of beta-secretase by p25/cdk5 leads to enhanced amyloidogenic processing.* Neuron, 2008. **57**(5): p. 680-90.

133. Sadleir, K.R. and R. Vassar, *Cdk5 protein inhibition and Abeta42 increase BACE1 protein level in primary neurons by a post-transcriptional mechanism: implications of CDK5 as a therapeutic target for Alzheimer disease.* J Biol Chem, 2012. **287**(10): p. 7224-35.

134. MacCallum, D.E., et al., *Seliciclib (CYC202, R-Roscovitine) induces cell death in multiple myeloma cells by inhibition of RNA polymerase II-dependent transcription and down-regulation of Mcl-1.* Cancer Res, 2005. **65**(12): p. 5399-407.

135. Noel, S., et al., *Discovery of pyrrolo[2,3-b]pyrazines derivatives as submicromolar affinity activators of wild type, G551D, and F508del cystic fibrosis transmembrane conductance regulator chloride channels.* J Pharmacol Exp Ther, 2006. **319**(1): p. 349-59.

136. He, M., et al., *Roscovitine attenuates intimal hyperplasia via inhibiting NF-kappaB and STAT3 activation induced by TNF-alpha in vascular smooth muscle cells.* Biochem Pharmacol, 2017. **137**: p. 51-60.

137. Whittaker, S.R., et al., *The Cyclin-dependent kinase inhibitor CYC202 (R-roscovitine) inhibits retinoblastoma protein phosphorylation, causes loss of Cyclin D1, and activates the mitogen-activated protein kinase pathway.* Cancer Res, 2004. **64**(1): p. 262-72.

138. Mandl, M.M., et al., *Inhibition of Cdk5 induces cell death of tumor-initiating cells.* Br J Cancer, 2017. **116**(7): p. 912-922.

139. Ray, B., et al., *Human primary mixed brain cultures: preparation, differentiation, characterization and application to neuroscience research.* Mol Brain, 2014. **7**: p. 63.

140. Masliah, E., et al., *Altered expression of synaptic proteins occurs early during progression of Alzheimer's disease.* Neurology, 2001. **56**(1): p. 127-9.

141. Alley, G.M., et al., *Memantine lowers amyloid-beta peptide levels in neuronal cultures and in APP/PS1 transgenic mice.* J Neurosci Res, 2010. **88**(1): p. 143-54.

142. Ray, B., et al., *Human primary mixed brain cultures: preparation, long-term maintenance, characterization and application to neuroscience research.* Mol Brain, 2014. **7**(1): p. 63.

143. Hagenbuchner, J. and M.J. Ausserlechner, *Targeting transcription factors by small compounds--Current strategies and future implications.* Biochem Pharmacol, 2016. **107**: p. 1-13.

144. Taylor, R.C. and A. Dillin, *Aging as an event of proteostasis collapse.* Cold Spring Harb Perspect Biol, 2011. **3**(5).

145. Motonaga, K., et al., *Up-regulation of E2F-1 in Down's syndrome brain exhibiting neuropathological features of Alzheimer-type dementia.* Brain Res, 2001. **905**(1-2): p. 250-3.

146. Ranganathan, S., S. Scudiere, and R. Bowser, *Hyperphosphorylation of the retinoblastoma gene product and altered subcellular distribution of E2F-1 during Alzheimer's disease and amyotrophic lateral sclerosis.* J Alzheimers Dis, 2001. **3**(4): p. 377-385.

147. Jantaratnotai, N., et al., *Upregulation and expression patterns of the angiogenic transcription factor ets-1 in Alzheimer's disease brain.* J Alzheimers Dis, 2013. **37**(2): p. 367-77.

148. Bourtchuladze, R., et al., *Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein.* Cell, 1994. **79**(1): p. 59-68.

149. Ettcheto, M., et al., *Early Preclinical Changes in Hippocampal CREB-Binding Protein Expression in a Mouse Model of Familial Alzheimer's Disease.* Mol Neurobiol, 2017.

150. Smith, T.G., P.A. Robbins, and P.J. Ratcliffe, *The human side of hypoxia-inducible factor.* Br J Haematol, 2008. **141**(3): p. 325-34.

151. Ashok, B.S., T.A. Ajith, and S. Sivanesan, *Hypoxia-inducible factors as neuroprotective agent in Alzheimer's disease.* Clin Exp Pharmacol Physiol, 2017. **44**(3): p. 327-334.

152. Rojanathammanee, L., et al., *Attenuation of microglial activation in a mouse model of Alzheimer's disease via NFAT inhibition.* J Neuroinflammation, 2015. **12**: p. 42.

153. Zhao, T., et al., *Age-related increases in amyloid beta and membrane attack complex: evidence of inflammasome activation in the rodent eye.* J Neuroinflammation, 2015. **12**: p. 121.

154. Ueberham, U., et al., *Altered subcellular location of phosphorylated Smads in Alzheimer's disease.* Eur J Neurosci, 2006. **24**(8): p. 2327-34.

155. Chalmers, K.A. and S. Love, *Neurofibrillary tangles may interfere with Smad 2/3 signaling in neurons.* J Neuropathol Exp Neurol, 2007. **66**(2): p. 158-67.

156. Song, W. and D.K. Lahiri, *Isolation of the genomic clone of the rhesus monkey beta-amyloid precursor protein.* Biochem Mol Biol Int, 1998. **46**(4): p. 755-64.

157. Saykin, A.J., et al., *Genetic studies of quantitative MCI and AD phenotypes in ADNI: Progress, opportunities, and plans.* Alzheimers Dement, 2015. **11**(7): p. 792-814.

158. Velayudhan, L., et al., *Entorhinal cortex thickness predicts cognitive decline in Alzheimer's disease.* J Alzheimers Dis, 2013. **33**(3): p. 755-66.

159. Burggren, A.C., et al., *Thickness in entorhinal and subicular cortex predicts episodic memory decline in mild cognitive impairment.* Int J Alzheimers Dis, 2011. **2011**: p. 956053.

160. Lee, M.L., et al., *Brain endothelial cells induce astrocytic expression of the glutamate transporter GLT-1 by a Notch-dependent mechanism.* J Neurochem, 2017.

161. Adwan, L., et al., *Tolfenamic acid reduces tau and CDK5 levels: implications for dementia and tauopathies.* J Neurochem, 2015. **133**(2): p. 266-72.

162. Ban, H.S., et al., *Identification of HSP60 as a primary target of o-carboranylphenoxyacetanilide, an HIF-1alpha inhibitor.* J Am Chem Soc, 2010. **132**(34): p. 11870-1.

163. Huang, W., et al., *Small-molecule inhibitors targeting the DNA-binding domain of STAT3 suppress tumor growth, metastasis and STAT3 target gene expression in vivo.* Oncogene, 2016. **35**(6): p. 783-92.

164. Delgado-Morales, R., et al., *Epigenetic mechanisms during ageing and neurogenesis as novel therapeutic avenues in human brain disorders.* Clin Epigenetics, 2017. **9**: p. 67.

165. Modgil, S., et al., *Role of early life exposure and environment on neurodegeneration: implications on brain disorders.* Transl Neurodegener, 2014. **3**: p. 9.

166. Cannon, J.R. and J.T. Greenamyre, *The role of environmental exposures in neurodegeneration and neurodegenerative diseases.* Toxicol Sci, 2011. **124**(2): p. 225-50.

167. Lahiri, D.K. and B. Maloney, *The "LEARn" (latent early-life associated regulation) model: an epigenetic pathway linking metabolic and cognitive disorders.* J Alzheimers Dis, 2012. **30 Suppl 2**: p. S15-30.

168. Maloney, B., et al., *Applying epigenetics to Alzheimer's disease via the latent early-life associated regulation (LEARn) model.* Curr Alzheimer Res, 2012. **9**(5): p. 589-99.

169. Henikoff, S. and M.A. Matzke, *Exploring and explaining epigenetic effects.* Trends Genet, 1997. **13**(8): p. 293-5.

170. Peleg, S., et al., *Altered histone acetylation is associated with age-dependent memory impairment in mice.* Science, 2010. **328**(5979): p. 753-6.

171. Rao, J.S., et al., *Epigenetic modifications in frontal cortex from Alzheimer's disease and bipolar disorder patients.* Transl Psychiatry, 2012. **2**: p. e132.

172. Ziller, M.J., et al., *Charting a dynamic DNA methylation landscape of the human genome.* Nature, 2013. **500**(7463): p. 477-81.

173. Brewer, G.J. and J.R. Torricelli, *Isolation and culture of adult neurons and neurospheres.* Nat Protoc, 2007. **2**(6): p. 1490-8.

174. Long, J.M., B. Ray, and D.K. Lahiri, *MicroRNA-153 physiologically inhibits expression of amyloid-beta precursor protein in cultured human fetal brain cells and is dysregulated in a subset of Alzheimer disease patients.* J Biol Chem, 2012. **287**(37): p. 31298-310.

175. Reynolds, B.A., W. Tetzlaff, and S. Weiss, *A multipotent EGF-responsive striatal embryonic progenitor cell produces neurons and astrocytes.* J Neurosci, 1992. **12**(11): p. 4565-74.

176. Reynolds, B.A. and S. Weiss, *Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system.* Science, 1992. **255**(5052): p. 1707-10.

177. Gritti, A., et al., *Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain.* J Neurosci, 1999. **19**(9): p. 3287-97.

178. Gritti, A., et al., *Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor.* J Neurosci, 1996. **16**(3): p. 1091-100.

179. Gritti, A., et al., *Basic fibroblast growth factor supports the proliferation of epidermal growth factor-generated neuronal precursor cells of the adult mouse CNS.* Neurosci Lett, 1995. **185**(3): p. 151-4.

180. Reynolds, B.A. and S. Weiss, *Clonal and population analyses demonstrate that an EGF-responsive mammalian embryonic CNS precursor is a stem cell.* Dev Biol, 1996. **175**(1): p. 1-13.

181. Salih, D.A. and A. Brunet, *FoxO transcription factors in the maintenance of cellular homeostasis during aging.* Curr Opin Cell Biol, 2008. **20**(2): p. 126-36.

182. Hoekman, M.F., et al., *Spatial and temporal expression of FoxO transcription factors in the developing and adult murine brain.* Gene Expr Patterns, 2006. **6**(2): p. 134-40.

183. Meijer, L. and E. Raymond, *Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials.* Acc Chem Res, 2003. **36**(6): p. 417-25.

184. Calnan, D.R., et al., *Methylation by Set9 modulates FoxO3 stability and transcriptional activity.* Aging (Albany NY), 2012. **4**(7): p. 462-79.

185. Sengupta, D., et al., *DNA methylation and not H3K4 trimethylation dictates the expression status of miR-152 gene which inhibits migration of breast cancer cells via DNMT1/CDH1 loop.* Exp Cell Res, 2016. **346**(2): p. 176-87.

186. Li, N., et al., *Epigenetic silencing of MicroRNA-503 regulates FANCA expression in non-small cell lung cancer cell.* Biochem Biophys Res Commun, 2014. **444**(4): p. 611-6.

187. Zhou, B., et al., *MicroRNA-503 targets FGF2 and VEGFA and inhibits tumor angiogenesis and growth.* Cancer Lett, 2013. **333**(2): p. 159-69.

188. Cao, C., et al., *miR-125b targets DNMT3b and mediates p53 DNA methylation involving in the vascular smooth muscle cells proliferation induced by homocysteine.* Exp Cell Res, 2016. **347**(1): p. 95-104.

189. Goeppert, B., et al., *Cadherin-6 is a putative tumor suppressor and target of epigenetically dysregulated miR-429 in cholangiocarcinoma.* Epigenetics, 2016: p. 0.

190. Samuel, N., et al., *Genome-Wide DNA Methylation Analysis Reveals Epigenetic Dysregulation of MicroRNA-34A in TP53-Associated Cancer Susceptibility.* J Clin Oncol, 2016.

191. Kaur, S., et al., *MicroRNA Methylation in Colorectal Cancer.* Adv Exp Med Biol, 2016. **937**: p. 109-22.

192. Maloney, B. and D.K. Lahiri, *Epigenetics of dementia: understanding the disease as a transformation rather than a state.* Lancet Neurol, 2016. **15**(7): p. 760-774.

193. Piletic, K. and T. Kunej, *MicroRNA epigenetic signatures in human disease.* Arch Toxicol, 2016.

194. Lahiri, D.K., B. Maloney, and N.H. Zawia, *The LEARn model: an epigenetic explanation for idiopathic neurobiological diseases.* Mol Psychiatry, 2009. **14**(11): p. 992-1003.

195. Shields, A.E., et al., *Childhood abuse, promoter methylation of leukocyte NR3C1 and the potential modifying effect of emotional support.* Epigenomics, 2016.

196. Provenzi, L., et al., *SLC6A4 methylation as an epigenetic marker of life adversity exposures in humans: A systematic review of literature.* Neurosci Biobehav Rev, 2016. **71**: p. 7-20.

197. Doura, M.B. and E.M. Unterwald, *MicroRNAs Modulate Interactions between Stress and Risk for Cocaine Addiction.* Front Cell Neurosci, 2016. **10**: p. 125.

198. Wingo, A.P., et al., *Genome-wide association study of positive emotion identifies a genetic variant and a role for microRNAs.* Mol Psychiatry, 2016.

199. Meydan, C., S. Shenhar-Tsarfaty, and H. Soreq, *MicroRNA Regulators of Anxiety and Metabolic Disorders.* Trends Mol Med, 2016. **22**(9): p. 798-812.

200. Jin, J., et al., *miR-17-92 Cluster Regulates Adult Hippocampal Neurogenesis, Anxiety, and Depression.* Cell Rep, 2016. **16**(6): p. 1653-63.

201. Vaisvaser, S., et al., *Neuro-Epigenetic Indications of Acute Stress Response in Humans: The Case of MicroRNA-29c.* PLoS One, 2016. **11**(1): p. e0146236.

202. Zhang, Y.L., et al., *Anxiety-like behavior and dysregulation of miR-34a in triple transgenic mice of Alzheimer's disease.* Eur Rev Med Pharmacol Sci, 2016. **20**(13): p. 2853-62.

203. Short, A.K., et al., *Elevated paternal glucocorticoid exposure alters the small noncoding RNA profile in sperm and modifies anxiety and depressive phenotypes in the offspring.* Transl Psychiatry, 2016. **6**(6): p. e837.

204. Capell, A., et al., *Maturation and pro-peptide cleavage of beta-secretase.* J Biol Chem, 2000. **275**(40): p. 30849-54.

205. Walter, J., et al., *Phosphorylation regulates intracellular trafficking of beta-secretase.* J Biol Chem, 2001. **276**(18): p. 14634-41.

206. Cole, S.L. and R. Vassar, *The Alzheimer's disease beta-secretase enzyme, BACE1.* Mol Neurodegener, 2007. **2**: p. 22.

207. Thinakaran, G. and E.H. Koo, *Amyloid precursor protein trafficking, processing, and function.* J Biol Chem, 2008. **283**(44): p. 29615-9.

208. Yan, R., et al., *The transmembrane domain of the Alzheimer's beta-secretase (BACE1) determines its late Golgi localization and access to beta -amyloid precursor protein (APP) substrate.* J Biol Chem, 2001. **276**(39): p. 36788-96.

209. Brown, M.S., et al., *Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans.* Cell, 2000. **100**(4): p. 391-8.

210. Iwatsubo, T., *The gamma-secretase complex: machinery for intramembrane proteolysis.* Curr Opin Neurobiol, 2004. **14**(3): p. 379-83.

211. Benjannet, S., et al., *Post-translational processing of beta-secretase (beta-amyloid-converting enzyme) and its ectodomain shedding. The pro- and transmembrane/cytosolic domains affect its cellular activity and amyloid-beta production.* J Biol Chem, 2001. **276**(14): p. 10879-87.

212. Cook, D.G., et al., *Alzheimer's A beta(1-42) is generated in the endoplasmic reticulum/intermediate compartment of NT2N cells.* Nat Med, 1997. **3**(9): p. 1021-3.

213. Hartmann, T., et al., *Distinct sites of intracellular production for Alzheimer's disease A beta40/42 amyloid peptides.* Nat Med, 1997. **3**(9): p. 1016-20.

214. Lieu, Z.Z. and P.A. Gleeson, *Endosome-to-Golgi transport pathways in physiological processes.* Histol Histopathol, 2011. **26**(3): p. 395-408.

215. Koh, Y.H., et al., *BACE is degraded via the lysosomal pathway.* J Biol Chem, 2005. **280**(37): p. 32499-504.

216. Huse, J.T., et al., *Maturation and endosomal targeting of beta-site amyloid precursor protein-cleaving enzyme. The Alzheimer's disease beta-secretase.* J Biol Chem, 2000. **275**(43): p. 33729-37.

217. Seaman, M.N., *The retromer complex - endosomal protein recycling and beyond.* J Cell Sci, 2012. **125**(Pt 20): p. 4693-702.

218. Small, S.A., et al., *Model-guided microarray implicates the retromer complex in Alzheimer's disease.* Ann Neurol, 2005. **58**(6): p. 909-19.

219. Nordberg, M., et al., *Lead concentrations in elderly urban people related to blood pressure and mental performance: results from a population-based study.* Am J Ind Med, 2000. **38**(3): p. 290-4.

220. Bihaqi, S.W., et al., *Infantile exposure to lead and late-age cognitive decline: relevance to AD.* Alzheimers Dement, 2014. **10**(2): p. 187-95.

221. Wu, J., et al., *Alzheimer's disease (AD)-like pathology in aged monkeys after infantile exposure to environmental metal lead (Pb): evidence for a developmental origin and environmental link for AD.* J Neurosci, 2008. **28**(1): p. 3-9.

CURRICULUM VITAE

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Education

2017	Indiana University, Indianapolis. Ph.D. in Medical & Molecular Genetics with minor in Life Sciences. Advisor: Debomoy K. Lahiri, Ph.D.
2003	Indiana University, Bloomington, B.S. in Biology

Awards

2017	Indy's Best & Brightest Award Finalist, Junior Achievement of Central Indiana
2017	Women of Influence Advocate Trailblazer Award, Indiana University National Center of Excellence in Women's Health
2017	Alzheimer's Association International Conference Travel Fellowship Award
2017	Exchange Leadership Fellow, Indianapolis Urban League
2017	IUPUI William M. Plater Civic Medallion Recipient, Indiana University Purdue University-Indianapolis
2017	Indianapolis Business Journal Forty Under 40
2017	Elite 50, Indiana University Purdue University-Indianapolis
2016	Dr. George Rawls Emerging Leader Award, 100 Black Men of Indianapolis
2016	Center for Leadership Development Achievers "Science & Technical Disciplines" Award

2015 President's Diversity Dissertation Fellowship Award, Indiana University Purdue University Indianapolis

Publications

- **Bayon BL**, Maloney B, Zawia N, Lahiri DK. (2017) Leading the Lead (Pb) Crisis with the Four Rs: Restoration, Renovation, Research and Remediation. *Environmental Health Perspectives*. Submitted.
- Erickson, CA, Wink L, **Bayon BL**, Ray B, Schaefer T, Pedapati E, Lahiri DK. (2016) Analysis of Peripheral Amyloid Precursor Protein in Angelman Syndrome. *Am J Med Genet* Part A 9999A:1-4.
- Lahiri DK, Maloney B, **Bayon BL**, Chopra N, White F, Greig NH, Nurnberger JI. (2016) Transgenerational Latent Early-life Associated Regulation (tLEARn): Uniting environment, life exposures, and genetic inheritance across generations. *Epigenomics* 8(3):373-87
- Srinivasan M, Chopra N, **Bayon BL**, Lahiri DK. (2016) Novel Nuclear Factor-KappaB Targeting Peptide Suppresses β-Amyloid Induced Inflammatory and Apoptotic Responses in Neuronal Cells. *PLoS One* 11(10):e0160314.
- Erickson CA, Ray B, Wink LK, **Bayon BL**, Pedapati EV, Shaffer R, Schaeffer TL, Lahiri DK. (2016) Initial analysis of peripheral lymphocytic extracellular signal related kinase activation in autism. *Journal of Psychiatric Research* 84:153-160

Abstracts and Presentations

- **Bayon BL**, Maloney B, Xu X-M, Ratan RR, Lahiri DK. (2017) The role of Sp1-modulating compounds in Alzheimer's disease. *Abstract accepted.* Society for Neuroscience Annual Meeting, Washington, D.C. Poster presentation.
- **Bayon BL**, Maloney B, Chopra N, White FA, Xu X-M, Ratan RR, Lahiri DK. (2017) Sp1-Modulating Compounds As a Novel Drug Target for Alzheimer's Disease (AD). Alzheimer's Association International Conference, London, United Kingdom. Oral Presentation. O4-06-01 *#18784*.
- **Bayon BL**, Nho K, Saykin AJ, Srinivasan M, Lahiri DK. (2017) Role of SP1 and Other Transcription Factors in Alzheimer's Disease. Alzheimer's Association International Conference, London, United Kingdom. Poster P4-02 *#18858*
- **Bayon BL**, Nho K, Maloney B, Chopra N, Lahiri DK. (2016) Regulation of amyloid-beta precursor protein (APP) and beta-secretase 1 (BACE1) expression by transcription factor modulating compounds mithramycin A and tolfenamic acid in human cells. *Abstract* from Society for Neuroscience Annual Meeting, San Diego, California. Presentation 785.20
- **Bayon BL**, Nho K, Maloney B, Chopra N, Lahiri DK. (2016) Differential regulation of amyloid-β precursor protein (APP) and β-secretase 1 (BACE1) by transcription factor (TF) modulating drugs in human cells. Presented at the 66th Annual Meeting of The American Society of Human Genetics, Vancouver, British Columbia, Canada. Poster 2040

- **Bayon, BL**, Nho, K, Maloney, B, Chopra, N, Lahiri, DK. (2015). Transcription factor mediated modulation of amyloid-beta precursor protein (APP) and beta-site APP cleaving enzyme (BACE1) expression as a novel drug target in Alzheimer's disease (AD). *Abstract* from Society for Neuroscience Annual Meeting, Chicago, Illinois. Presentation 214.09/C8
- Lahiri, DK, Maloney, B, Long, JM, Chopra, N, Sambamurti, B, **Bayon, BL**. (2015). Understanding the neurobiology of Alzheimer's disease (AD) by correlating specific AD-associated miRNAs and the MMSE cognitive scale. *Abstract* from Society for Neuroscience Annual Meeting, Chicago, Illinois. Presentation 214.10/C9
- **Bayon, BL**, Nho, K, Maloney, B, Chopra, N, Lahiri, DK. (2015). Transcription factor mediated modulation of amyloid-beta precursor protein (APP) and beta-site APP cleaving enzyme (BACE1) expression as a novel drug target in Alzheimer's disease (AD). Presented at the 65th Annual Meeting of The American Society of Human Genetics, Baltimore, Maryland. Poster 1218T.
- **Bayon, BL**, Bailey, JA, Ray, B, Sambamurti, K, Greig, N, Lahiri, DK. (2015). Role of Sp1 inhibiting drugs in the modulation of amyloid-beta precursor protein (APP) and beta-site APP cleaving enzyme (BACE1) activity in human cells: implications as a novel drug target for Alzheimer's disease. *Abstract* from Indiana Clinical and Translational Sciences Institute (CTSI) Annual Meeting, Indianapolis, Indiana.

- **Bayon, BL**, Bailey, JA, Ray, B, Sambamurti, K, Greig, N, Lahiri, DK. (2014). SP1 inhibitors as modulators of APP and BACE1 levels in human cells: A novel drug target in Alzheimer's disease. *Abstract* from American Society of Human Genetics Annual Meeting, San Diego, California. Poster 1354S.
- **Bayon, BL**, Bailey, JA, Ray, B, Sambamurti, K, Greig, N, Lahiri, DK. (2014). SP1 inhibitors as modulators of APP and BACE1 levels in human cells: A novel drug target in Alzheimer's disease. *Abstract* from Drug Discovery and Therapy World Congress, Boston, Massachusetts. Session Lecture SL-184(a). Poster PO-90.
- **Bayon, BL**, Lahiri, DK, Bailey, JA. (2013). BACE1 gene regulation: A novel drug target in Alzheimer's disease. Alzheimer's & Dementia, 2013. **9**(4, Supplement): p. P304. *Abstract* from Alzheimer's Association International Conference, Boston, Massachusetts.
- **Bayon, BL**, Bailey, JA, Lahiri, DK (2013). BACE1 Gene Regulation: A Novel Drug Target in Alzheimer's disease. Poster session presented at IUPUI Research Day 2013, Indianapolis, Indiana. https://scholarworks.iupui.edu/handle/1805/6938
- **Bayon, BL**, Celerin, M, Zolan, M (2001). Mutation in the msh5 Gene of 0-376 in Coprinus cinereus likely causes an N-Terminus Truncation of the Protein. *Abstract* from Annual Biomedical Research Conference for Minority Students (ABRCMS). Orlando, Florida.

Societies and Organizations

Board of Directors, Adult & Child Health, Indianapolis **2016 - present**

Board of Directors, TechIndy STEM High School, Indianapolis **2016**

Social Media Secretary, Midwest Africa Chamber of Commerce **2016**

Mentor, 100 Black Men of Indianapolis “Financial Literacy Program” **2015-present**

Society for Neuroscience “Neuroscience Scholars Program Associate” **2015-present**

Member, The American Society of Human Genetics, **2014 – present**

Member, Society for Neuroscience, **2013 – present**

Member, National Organization for the Professional Advancement of Black Chemists and Chemical Engineers, **2013 – present**

Mentor, College Prep Academy, St. Vincent Hospital **2012**

Member, Alpha Chi Sigma, **2002-present**