NEURODEGENERATIVE MODELING: TAU PROTEIN, DEGRADATIVE PATHWAYS, AND GENE EXPRESSION PROFILING OF HUMAN IPSC-DERIVED NEURAL PRECURSORS AND DIFFERENTIATED 3-D NEURAL SPHERE VERSUS 2-D MONOLAYER CULTURES

By

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ABSTRACT

NEURODEGENERATIVE MODELING: TAU PROTEIN, DEGRADATIVE PATHWAYS, AND GENE EXPRESSION PROFILING OF HUMAN IPSC-DERIVED NEURAL PRECURSORS AND DIFFERENTIATED 3-D NEURAL SPHERE VERSUS 2-D MONOLAYER CULTURES

Kyle H. Anthoney

Human induced pluripotent stem cells offer a model for human brain development and disease by differentiation into brain organoids; however, current neural culture systems lack the microenvironment, neuronal circuits and connectivity, vascular circulation, and immune system that exist in vivo. After differentiation and development of neuronal and non-neuronal cell types within two formats of cell cultures, we can visualize and recapitulate in vivo protein accumulation, gene expression, and degradative processes such as autophagy. Using RNA extraction, purification methods and reverse transcription I compared traditional monolayer cultures and novel 3-D neural sphere cultures via gene expression analysis. This analysis indicated variable gene expression between formats therefore only monolayer cultures were analyzed for tau protein accumulation with pharmacologic treatments and measured by Western blot. I also report on cell type specific gene expression by transient transfection of plasmid cassette tools and fluorescent microscopy. Here, cell type specific gene targeting is demonstrated in successfully transfected cells. Further development of the tools utilized in this study will significantly expand the field of neurodegenerative research, by giving us the ability to
target specific cell types within mixed cultures, and allowing for a more accurate
depiction of pathogenesis within diseased cell types.
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Figure 27. Neural cells after transfection with housekeeping EEF1A1-GFP plasmid cassette. Fluorescent microscopy reveals EGFP expression in iPSC derived neural cells. (TOP) Cells transfected with plasmid cassettes containing EGFP sequences induced by EEF1A1 reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows NPCs at day 0 of differentiation, 24 hours after transfection. Lane 2 shows nine days differentiated. Lane 3 shows 21 days differentiated. (BOTTOM) Enlarged image of differentiation day 21 displays cells expressing GFP. Red arrow shows possible neuronal cell type; red circle shows possible astrocyte cell types.

Figure 28. Neural cells after transfection with astrocytic GFAP-GFP plasmid cassette. Fluorescent microscopy reveals EGFP expression in iPSC derived neural cells. (TOP) Cells transfected with plasmid cassettes containing EGFP sequences induced by GFAP reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows cells at day 2 of differentiation. Lane 2 shows nine days differentiated. Lane 3 shows 21 days differentiated. Lane 4 shows 28 days differentiated. (BOTTOM) Enlarged image of differentiation day 9 exhibits cells expressing EGFP with a morphology resembling astrocytes.

Figure 29. Neural cells after transfection with oligodendrocytic CNP-GFP plasmid cassette. Fluorescent microscopy reveals extremely low levels of EGFP expression in iPSC derived neural cells. (TOP) Cells transfected with plasmid cassettes containing EGFP sequences induced by CNP reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows cells at day 2 of differentiation. Lane 2 shows nine days differentiated. Lane 3 shows 18 days differentiated. (BOTTOM) Enlarged image of differentiation day 9 exhibits cells expressing EGFP. Cell types cannot be confirmed.
Figure 30. Neural cells after transfection with Neuronal SYN1-GFP plasmid cassette. Fluorescent microscopy reveals relatively low levels of EGFP expression in iPSC derived neural cells. Cells transfected with plasmid cassettes containing EGFP sequences induced by SYN1 reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows cells at day 2 of differentiation. Lane 2 shows 9 days differentiated. Lane 3 shows 18 days differentiated. Enlarged images (indicated by red lines) exhibit cells expressing EGFP after 18 days of differentiation.

Figure 31. Neural cells after transfection with neuronal PRNP-GFP plasmid cassette. Fluorescent microscopy reveals relatively high levels of EGFP expression in iPSC derived neural cells. Cells transfected with plasmid cassettes containing EGFP sequences induced by SYN1 reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. (TOP) Lane 1 shows cells at day 2 of differentiation. Lane 2 shows 9 days differentiated. Lane 3 shows 28 days differentiated. Lane 4 shows 34 days differentiated. (BOTTOM) Enlarged image exhibits cells expressing EGFP after 34 days of differentiation.

Figure 32. Neural cells after transfection with no plasmid cassette. Fluorescent microscopy reveals some EGFP expression in iPSC derived neural cells. After “transfection”, cells were subsequently differentiated by FGF withdrawal. Lane 1, cells at day 21 of differentiation. Lane 2, 25 days differentiated. Lane 3, 28 days differentiated.

Figure 33. Bright field micrographs of 2D monolayer (left) and 3-D neural spheres (right). Cells displayed here were cultured from CV17q iPSC derived neural progenitor cells and further differentiated. (Left) Bright field microscopy shows the monolayer cell cultures, traditionally used in neural developmental research. (Right) Bright field microscopy showing different growth stages of differentiated neural spheres. Axons and dendrites can be more easily distinguished in the 3-D cultures as well as obvious connections that are being made between cells.
INTRODUCTION

Microtubule associated protein tau (MAPT) has been implicated in a specific class of neurodegenerative diseases called tauopathies which leave millions devastated worldwide (Larsen et al. 2015). Tauopathies are characterized by specific isoforms of misfolded, insoluble, hyperphosphorylated tau protein which aggregate as neurofibrillary tangles in the brain (Iqbal et al. 2010). The MAPT gene encodes the tau protein where six different splice variants exist in the adult human brain. Criteria used for classification of these diseases include protein binding domains and/or number of N-terminal domains. Contingent upon exon 10, tau is first identified as 3 (3R) or 4 (4R) repeats by the number of microtubule binding domains. 3R is a "signpost" for tauopathies such as Myotonic dystrophy and Pick’s disease. 4R tau is indicative of several other tauopathies: corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and argyrophilic grain disease (AGD); sporadic predominant primary tauopathies that accumulate hyperphosphorylated tau within neurons, oligodendrocytes, and astrocytes. While different ratios also categorize these diseases (de Silva et al. 2006), differences between isoforms, as well as cell type (neurons vs. glia) in which aggregation occurs, may be attributed to the gene expression patterns and primary function of MAPT. Isoforms are classified further by 0, 1, or 2 N-terminal domains (Iqbal et al. 2010). A critical question in the study of tauopathies is how abnormal expression of MAPT is managed by neurons, astrocytes, and oligodendrocytes, and which pathways are required to maintain viability when MAPT gene products accumulate.
Degradative Pathways

The Ubiquitin proteasome system (UPS) and autophagy pathway use ubiquitylation as a signal to coordinate and localize (protein) cargo for degradation. The proteasome prevents the accumulation of misfolded proteins, influences the cell cycle, and regulates the immune response (Tai and Schumann 2008). The UPS has a size limitation for molecules that may be degraded. When this system becomes impaired or a substrate cannot fit, UPS inhibition induces autophagy as an auxiliary system which then non-selectively clears protein (Tang et al. 2014, Lotfy 2016, Bose and Cho 2017). Non-native protein conformations are usually selectively recognized by chaperones and undergo repair or refolding. If chaperone repairing activity fails to execute, the altered proteins are processed to the UPS for degradation (Martini-Stoica et al. 2016). However, if the UPS functionality is impaired or if the protein has already generated insoluble structures, autophagy is the only option for complexed toxic protein removal (Bose and Cho 2017). Autophagy is a system that delivers cytoplasmic components, tagged by ubiquitin, to lysosomes for terminal degradation and dysfunction of this pathway is hypothesized to play a key role in the pathogenesis of several neurodegenerative diseases (Nedelsky et al. 2008). In the autophagy pathway, autophagosomes are formed by sequential functions of autophagy-related (ATG) proteins. Microtubule-associated protein light chain 3 (LC3) is required in the recruitment of substrates to the autophagosome (Martini-Stoica et al. 2016). The cytosolic form of LC3 known here as LC3-I, becomes conjugated with the phospholipid, phosphatidylethanolamine and results in a lipid
modified-LC3 known as LC3-II. This LC3-II associates with the autophagosome membrane and then sequester its cargo within the autophagosome for degradation. Studies of upstream signaling pathways, autophagosome formation, and autophagosome maturation/fusion with lysosomes are critical in addressing which isoform(s) of tau are present in each cell, which isoform(s) are signaled for degradation and by which pathway, as well as understanding autophagy clearance and how tauopathies manifest in human brain cells.

Modeling Tauopathies

Misfolded, insoluble tau proteins are characteristic of tauopathies and form the neurofibrillary tangles. Those who develop a neurodegenerative disease usually exhibit memory impairment associated with brain atrophy and neuronal loss due to presence of extracellular accumulation of plaques and neurofibrillary tangles in the brain.

Differentiation of induced pluripotent stem cells (iPSCs) has allowed us to study the overall development of many cell types and tissue layers as well as the underlying molecular mechanisms that activate and manage the development/construction of these elements. After somatic cell-nuclear transfer, in which allocation of nuclear content into oocytes is established, or by embryonic stem cell fusion, cell cultures can be reverted to embryonic-like cells (Takahashi and Yamanaka 2006). With the addition of appropriate signaling factors, iPSCs can be coaxed to yield eye, gut, liver, kidney, and other human tissues. Similar to these 3D multicellular structures, aggregates of iPSCs specific to populations of neural cells can assemble and self-organize. Indeed, various
interdependent and discrete brain regions develop to contain progenitor zone organization of human cortical neuronal subtypes termed cerebral organoids (Lancaster et al. 2013). Conservation of signal transduction and cellular connections should be an empirical intention/objective with iPSCs.

We can now study fluctuation in anatomical brain networks by focusing on brain network development, network structure, and network function where spatial and temporal factors have been demonstrated to shape connectome development (Kaiser 2017). Underlying molecular signals, spatial distance between cells and their environments, and the length of time when these signals are activated could influence neural development and synapse maturity (Cahoy et al. 2008). These factors also share importance in the influence of connections made between cells and the organization of signal pathways during brain development (Kaiser 2017). The visualization of tissue structures, based on gene expression, will provide us with contemporary measurements of spatial/temporal and functional development through cell signaling pathways.

Studies of MAPT knockout in mouse models have suggested that the MAPT gene has little to no effect on normal cell development and do not often recapitulate human disease especially in central nervous system models (van Hummel 2016). Mansour et al. (2018) hypothesized that intracerebral implantation of human stem cell derived brain organoids in mice could create an accommodating environment for organoid development and long-term survival. After transplantation into adult mouse brains, vascularization and connectome was established. Based on lentiviral transduction and expression of EGFP, grafts displayed neuronal differentiation and maturation,
gliogenesis, and microglia integration. Transgenic mouse models can recapitulate disease phenotypes, however there are concerns about the validity of these models; a number of genetic variants of human diseases are located in non-coding regions that are not conserved between species. With the advent of iPSC technology, pluripotent cell types have been used as an alternative way to model tauopathies (Raja et al. 2016). Transgenic studies could still provide insight into novel treatments for brain disorders or injuries however, since mouse and human models differ, the current experiment was performed solely on human cell lines for a more precise explication of human neural characterization.

Here I validate the CV17q cell line as a tauopathy model and CV4a line as a control. I used and discuss the derivation and differentiation of healthy and diseased iPSC lines in two formats. Induced pluripotent stem cells have been derived from a human donor with a fully published and sequenced genome (Israel et al. 2012). Craig Venter’s diploid genome has been sequenced in high resolution and made publicly available, and because he has not developed a neurodegenerative disease (at present time), CV4a clonally selected and differentiated neural progenitor cells were used as a healthy control. Based on single nucleotide polymorphism (SNP) array data from Jordan Dizon’s Master’s Thesis (2016), CV17 amplification (CV17q) cell line (derived from CV4a) contains a spontaneously amplified ~40Mbp sequence from the 17q chromosome to provide a 3rd copy of the MAPT gene. This amplification also includes several other genes which may be supplementary but irrelevant to this study. It is the consideration of the impact of tau expression and protein accumulation that authenticates CV17q to be a
model of tauopathy, though no pathology of any disease has yet been investigated.

The following specific aims address the central hypothesis. There is a gap in knowledge about the mechanisms of neural development, and the intricate roles of cell signaling, gene expression, and tau protein accumulation within diseased vs healthy cell types. MAPT gene products are implicated in the accumulation of toxic tau species in vulnerable cell types. The long-term goal of this project was early and targeted activation of the autophagy pathway to develop effective reiteration of in vivo processes using in vitro methodologies to promote specific clearance of pathogenic tau species. The identification and further analysis of fundamental gene expression and cell signaling is essential in exposing new diagnostic and discovery tools for the intervention/prevention of these diseases regarding the following central hypothesis that disruption of autophagy-essential protein components will produce disease-like cellular phenotypes and accelerated cell death in populations of human neural cultures. The overall objective was to determine the role of autophagy in the turnover of misfolded tau protein by human neural cells. Gene expression profiles in adherent vs 3-D neural sphere iPSC derived cell lines were also determined in support of the rationale that development of novel treatment strategies for intervention and/or prevention will alter the course of more than 14 clinically distinct neurological diseases.
Specific Aims

1. Evaluation of gene expression in 3-D neural sphere vs standard, adherent cell cultures.

   Stable and viable cells that have the potential of disease inheritance can be developed and scrutinized through neural progenitor differentiation using induced pluripotent stem cells with a cell line with a fully sequenced and published genome (Israel et al. 2012). Our iPSC lines were clonally selected and differentiated into neural progenitor cells from which brain cells can be further differentiated. Culture samples were developed in vitro by differentiating iPSCs into neurons, astrocytes, and oligodendrocytes which make up neural tissue of the CNS. Typically, cells are adhered to a surface and differentiated where restricted spatial factors establish connectome expansion. Compared with 2D sheets of neural cells, 3-D structures have an extended lifespan and can consist of heterogeneous populations. They also mimic key features of developing brains (Farahany et al. 2018). Primer sets target and amplify selected gene sequences where I hypothesized that expression of these genetic comparisons between adherent lines and 3-D non-adherent (neural sphere) lines would validate 3-D neural spheres as more accurate representations of in vivo physiological connectome and development.

2. Establishing cell-specific plasmid tools to target neurons, astrocytes, or oligodendrocytes within mixed cultures and label them with EGFP.

   To ascertain information about the development of brain cells, populations of cells were transfected with AAVS1 plasmid containing enhanced green fluorescent
protein (EGFP) induced by cell type specific reporter gene promoters (Figure 1) to visualize cells of interest. The detection of antibodies through fluorescent microscopy confirms cell type specific gene expression. Fluorescent images generate a map from which patterns of gene expression can further be organized. It was expected that these plasmid cassette tools would permit highly reproducible results in which different cell lines and their cell type specific gene expression may be evaluated.

Figure 1. Illustration of the AAVS1 donor plasmid including left and right homology arms, splice acceptor (SA), thiophene-2-acetate (T2A) cleavable linker, puromycin (Puro R) resistance. Post digestion with Sal I and inclusion of cell specific promoters allow EGFP expression in cell types of interest. Further digestion with ECORV and inclusion of CRISPR constructs to be activated via promoter within specific cell types. EEF1A1, GFAP, CNP, SYN1, OR PRNP were integrated into exclusive plasmid cassettes.

3. To determine the role of autophagosomes in the clearance and turnover of tau in brain cells.

Native tau is normally degraded by the UPS and if tau becomes misfolded, mechanisms are implemented to refold or degrade the protein. Misfolded tau protein is degraded primarily by the autophagy pathway and alternatively degraded via ubiquitin proteasome (Tang et al. 2014, Ciechanover and Kwon 2015, Bose and Cho 2017).

The proposed drug panel (Table 1) and molecular protein targeting implemented in this experiment distinguish tau degradation via autophagy from ubiquitin proteasome
degradation based on the levels of individual tau isoforms, total tau, and LC3 protein(s).

Activation of one or both degradative pathways stimulates the degradation of tau protein whereas inhibition of the same pathway(s) results in accumulation of protein and may be associated with the viability of each cell type. It has been established that drug regulation of LC3-II may elucidate autophagosome and ubiquitin binding inhibition, as well as other pathway mechanisms to investigate tau specific degradation.

Table 1. Pharmacologic pathway modulators. Compounds used to treat cell lines with known function related to cellular pathways and processes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Effect on Cellular Pathway(s)</th>
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</thead>
<tbody>
<tr>
<td>Rapamycin</td>
<td>mTOR Inhibitor-Autophagy Activator</td>
</tr>
<tr>
<td>Torin1</td>
<td>mTORC1/2 Inhibitor-Autophagy Activator</td>
</tr>
<tr>
<td>SCD1</td>
<td>Autophagy Inhibitor (inhibits autophagosome formation)</td>
</tr>
<tr>
<td>Spautin1</td>
<td>Autophagy Inhibitor (inhibits autophagosome formation)</td>
</tr>
<tr>
<td>Bafilomycin A1 (BafA1)</td>
<td>vATPase Inhibitor</td>
</tr>
<tr>
<td>Chloroquine (CQ)</td>
<td>Inhibitor Lysosomal Acidification</td>
</tr>
<tr>
<td>MG-132</td>
<td>UPS Inhibitor</td>
</tr>
<tr>
<td>Simvastatin</td>
<td>HMG-CoA Reductase Inhibitor (Cholesterol)</td>
</tr>
<tr>
<td>Nystatin</td>
<td>Lipid Raft Inhibitor (Ionophore)</td>
</tr>
<tr>
<td>FCCP</td>
<td>Mitochondrial Oxidative Phosphorylation Uncoupler</td>
</tr>
<tr>
<td>PuAD</td>
<td>HSP90 Inhibitor</td>
</tr>
<tr>
<td>Vorinostat</td>
<td>HDAC Inhibitor (non-selective)</td>
</tr>
</tbody>
</table>
MATERIALS AND METHODS

Cell Culture

Induced pluripotent stem cell lines were obtained from the Goldstein laboratory at University of California San Diego’s Sanford Consortium for Regenerative Medicine. Primary fibroblasts were previously extracted from patients via dermal punch biopsy and re-programmed to an embryonic-like state by transduction of supplemental signaling factors (Takahashi and Yamanaka 2006, Okita et al. 2007). Fibroblasts were transduced with vectors containing complementary DNA for OCT4, SOX2, KLF4, c-MYC and EGFP, and initiated cell fate lineages of ectodermal, mesodermal and endodermal cell types (Israel et al. 2012). Once pluripotency had been established, iPSC lines were differentiated into neural progenitor cultures (NPCs) containing neural rosettes (Wilson and Stice 2006, Israel et al. 2012) and then purified, as detailed below. Derived NPCs were differentiated for 3 weeks. Neural cells sequentially collected and purified by fluorescence activated cell sorting were assessed to be >90% neurons, based on present βIII-tubulin1, and MAP2 projections.

Adherent cells

Originating from J. Craig Venter, CV4a and CV17q iPSC cell lines had been derived through iPSC differentiation (Takahashi and Yamanaka 2006, Wilson and Stice 2006, Okita et al. 2007, Israel et al. 2012). These iPSC lines had been previously clonally selected with antigen markers and then differentiated into neural progenitor cells from which populations of brain cells were further differentiated.
Neural progenitor cells were thawed from freezing media (NPCb + 10% DMSO), washed with Hank's Balanced Salt Solution, and plated on PLO/Laminin (Sigma-Aldrich, Poly-L-Ornithine hydrobromide)/(Corning, Mouse Laminin Ref. 354232) coated plates (Fisher brand, Polystyrene tissue culture treated low evaporation lid, flat bottom plates Ref. FB012927) in NPCb cell media (DMEM/F12 + GlutaMAX (Gibco; Ref. 10565-018), 1% Pen-Strep antibiotics (Gibco; Ref. 15140-122) 0.5x B-27 supplement (Gibco, Ref. 17504-044), 0.5x N-2 supplement (Gibco; Ref. 17502-048), and 20ng/ml Fibroblast Growth Factor (FGF PeproTech Animal-Free Recombinant Human FGF-basic, Ref. AF10018B100UG). Cells were grown to confluency and acutase (lot# 752919A) was used to dissociate the cells. Cells were counted on a hemocytometer and plated at >100,000 cells/ml. Passaged cells were re-adhered and differentiated from NPCs in NPCb following FGF withdrawal by media change. Media was changed once each week.

3-D neural spheres

Adhered NPCs were dissociated into single cell suspension using acutase. Cells were counted on a hemocytometer and resuspended in NPCb at >100,000 cells/ml. NPCs were transferred to non-coated plates, suspended in media containing 2ul/ml FGF, and shaken at 90RPMs on an orbital shaker. Cells were differentiated from NPCs in NPCb following FGF withdrawal by media change.

Differentiation and collection of NPCs/NSCs

2-D monolayer and 3D neural sphere cultures developed from NPCs in NPCb + FGF were replated and after successive FGF withdrawal, samples were collected every 4 days beginning at day 0 for 28 days and stored at -80°C.
Maintenance of cell cultures for drug treatments and protein analysis

NPCs were adhered to plates and grown to confluency as described previously. Cells were differentiated for 28 days via FGF withdrawal. Cells were dissociated and replated at a density of ~50,000 cells/well onto 96-well plates. Cells were counted using trypan blue to help determine cell viability.

Cell collection for Western blots and subsequent protein analysis

Based on cell viability assays, concentrations B and D (Table 2) were selected for protein analysis via Western blot. Cells were washed in PBS and lysed in buffer containing EDTA-Free Protease and Phosphatase inhibitors (Pierce, Mini Tablets 88669). Lysate protein concentrations were diluted to a maximum of 20µg/50µl. Samples with lower concentrations were collected in 40µl lysis buffer and 10µl 5x loading dye.

Reactions were boiled at 100°C for 10 minutes in a thermocycler.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Stock Conc</th>
<th>Working Conc A</th>
<th>Working Conc B</th>
<th>Working Conc C</th>
<th>Working Conc D</th>
</tr>
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<td>Rapamycin</td>
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<td>5.48µM</td>
<td>1.83µM</td>
<td>0.61µM</td>
<td>0.20µM</td>
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<tr>
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<td>1mM</td>
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<td>0.68µM</td>
<td>0.23µM</td>
<td>0.08µM</td>
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<td>Vorinostat</td>
<td>50mM</td>
<td>122µM</td>
<td>42.90µM</td>
<td>15.09µM</td>
<td>5.31µM</td>
</tr>
</tbody>
</table>

Table 2. Stock concentrations were initially diluted in NPCb and further diluted at 50µl treatments into 100µl NPCb. Table displays stock and diluted concentrations of each drug treatment. Color coded for drug concentration.
RNA Extraction and Supplemental DNase Treatment

Total RNA was obtained from cells via RNA extraction (Promega, ReliaPrep RNA tissue Miniprep System Ref. Z6111). Cells were washed in PBS (Gibco, Dulbecco's Phosphate Buffered Saline Ref. 14190-144), lysed in 1-thioglycerol solution, after which RNA was sequestered using 100% isopropanol. DNase treatment along with several RNA and column washes were performed according to the Promega protocol. There were slight deviations from the protocol in the number of cells collected and volume of lysis buffer used. RNA was eluted with DEPC treated molecular grade water (Ambion, P/N. AM9920, L/N. 1508030), where concentrations of ~15ng/ul and the 260/280 purity (1.7-2.1) were verified (Figure 2) using a Nanodrop (Thermoscientific, Nano Drop One). A second application of DNase (Promega, RQ1 RNase free DNase Ref. M610A) in 10x reaction buffer (Ref. M198A) was administered to all RNA samples (15ng/ul) that met criteria.

Figure 2. Measurements via Nanodrop provide RNA concentration (ng/ul), and purity in absorbance ratios at 260/280 and 260/230. Quantifications are displayed for three samples. Sample 1 was excluded from further experimental processing due to low concentration (ng/ul) and increased protein contamination (260/230 ratio).
Reverse Transcription

Synthesis of cDNA (Promega, GoScript Reverse Transcription System Ref. A5000) for both CV4a and CV17q cell lines per condition (2D vs 3-D) was achieved by annealing random hexamers, according to manufacturer instructions, to purified DNase treated RNA. Purified RNA was diluted to a concentration of 15ng/μl. A master mix was prepared in proper proportion(s): RNA, DEPC treated H₂O, GoScript 5x reaction buffer, 2.5mM MgCl₂, 0.5mM PCR Nucleotide mix, RNasin, and Goscript Reverse Transcriptase enzyme. A negative control mix was made to exclude the reverse transcriptase enzyme for validity of RNA, contaminant free from genomic DNA (Figure 3). All samples were placed in a Bio-Rad T100 thermocycler with annealing, extension, and inactivation temperatures of 25°C for 5 minutes, 42°C for one hour, and 70°C for 15 minutes, respectively. Final cDNA product was stored at -4°C.

**Figure 3.** EEF1A1 (lanes 2,3), RPL27 (lanes 4,5), MAPT-tot-2 (lanes 6,7), and ODZ2 (lanes 8,9) primer sets were used as an experimental control for reverse transcription. (A) PCR products loaded on 2% agarose gel in TBE. Lanes 3, 5, 7, 9 display bands where reverse transcriptase enzyme was excluded from the reaction indicating genomic DNA contamination. (B) Successful PCR amplification of positive RT enzyme Lanes 2, 4, 6, 8; and little to no amplification of genomic DNA in the negative control lanes 3, 5, 7, 9. Amplification of problematic cDNA samples and corresponding genes via PCR demonstrates a requirement for additional DNase treatment(s).
Primer Sets, Polymerase Chain Reaction and Gel Electrophoresis

Genes were selected based on bioinformatics from Cahoy et al. (2008). Primer sets were obtained through Harvard Primer bank (Table S1). Forward and reverse primers were re-suspended/diluted 1/20 in molecular grade H$_2$O. To avoid conjecture, primers have been screened via gel electrophoresis (Figure 4; Table S1). Primer sets were excluded/replaced if PCR products yielded bands that a) fell outside expected sequence length, b) largely generated primer dimers, or c) produced multiple band sizes. A master mix was made with 1ul cDNA, 7ul molecular grade H$_2$O, and 10ul 2x EconoTaq per reaction. 18ul master mix was amalgamated with 2ul combined forward and reverse primers diluted 1/20. cDNA was diluted with 100ul H$_2$O regardless of concentration. Reactions were completed in a Bio-Rad T100 thermocycler at 94°C, 52°C, and 72°C. Primers were prepared in 96-well deep well plates (Table 3). Agarose gels were prepared with 2% agarose (KSE Scientific, Molecular Biology Grade Agarose) in 1x TBE buffer. Gels were run at 100 volts for ~45 minutes, and visualized in Alpha imager (Alpha innotech, Multimage II version 3.5.0).
Figure 4. Gene placement map. Primers were selected based on cell specific gene expression. Primers were screened; yellow boxes and green arrows identify possible inconsistencies with primer sets. Could be non-specific binding or multiple isoforms/splice variants of those genes.
Table 3. Plate layout of forward and reverse primer sets. 2ul forward and 2ul reverse primers were prepared in 196ul molecular grade H$_2$O. Column 1 rows A-H contain MAPT primer sets. Column 2 rows A-D contain housekeeping gene primer sets. Column 2 rows E-H, Column 3 rows A-H, Column 4 A-B contain neuronal gene primer sets. Column 4 rows C-H, Column 5 rows A-H contain astrocytic gene primer sets. Column 6 rows A-H contain oligodendrocytic gene primer sets. Primer sequences are described in detail in the supplemental gene sequence Table S1.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
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<td>A</td>
<td>MAPT-tot</td>
<td>GAPDH</td>
<td>Syt1</td>
<td>Satb</td>
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<td>Gjb6</td>
<td>CNP</td>
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</table>
Cell Specific Gene Targeting with Reporter Plasmids

**HiFi assembly**

Each promoter sequence and EGFP was synthesized by PCR and inserted by HiFi assembly into the AAVS1 SA-2A-puro-pA donor plasmid at the SalI restriction site (Addgene plasmid # 22075; Hockemeyer et al. 2009). Each resulting plasmid was co-transfected with AAVS1-targeting sgRNA/Cas9-D10A dual nickase CRISPRs (px462, Addgene plasmid #62987) into neural progenitor cells, then selected for stable integration by puromycin. The following well-described synthetic gene promoters/enhancers (sourced from VectorBuilder, **Table 4**) were selected as cell specific markers: *PRNP* (all neurons, Weber et al. 2001); *SYN1* (mature neurons, Glover et al. 2002); *GFAP* (astrocytes, Brenner et al. 1994); *CNP* (oligodendrocytes/Schwann cells, Gravel et al. 1998); and *EEF1A1* (all cell types, Mizushima and Nagata, 1990). Cell type identity was confirmed by fluorescence microscopy (cell morphology) and by immunostaining with commonly used antibodies against these cell types (e.g. anti-GFAP, anti-Tuj1, anti-CNP, anti-tau etc.). Promoters, and PRP-TRE3G-EGFP-CV fragments were amplified separately via PCR (**Figure 5**). Promoter sequences (**Figure 5a**) together with EGFP-Cloning site fragments (**Figure 5b**) were HiFi assembled into the AAVS1-TRE vector backbone (**Figure 5c**). All DNA sequences (10ng/ul) were combined with the HiFi Assembly Master Mix according to manufacturer protocol (NE Builder HiFi DNA Assembly Cloning Kit, Master Mix; NEB #E2621) and incubated in a thermocycler at
50°C for 15 minutes. Samples were stored at -20°C. Once the plasmid was validated, the DNA was transformed into chemically-competent *E. coli* (Stbl3 cells).

**Table 4.** Primer sets used for plasmid construction. Promoter sequences, forward and reverse were used to amplify the promoter region of interest. Kozak sequences were used to amplify fully constructed plasmid cassettes.

<table>
<thead>
<tr>
<th>Reference #</th>
<th>Primer Name</th>
<th>Sequence</th>
<th>Tm (50mM NaCl)</th>
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<tr>
<td>194207966</td>
<td>EF1_Promoter_F</td>
<td>5’- GAT GCG GTG GGC TCT ATG GGG GCT CCG GTG CCC GTC -3’ (38bp)</td>
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<td>194207967</td>
<td>EF1_Promoter_R</td>
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<td>GFAP_Promoter_R</td>
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<td>CNP_KOZAK</td>
<td>5’- GAA CAC AAG CGC CAC CAT GGT GAG CAA G-3’ (31bp)</td>
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</table>

**Figure 5.** HiFi Assembly. (a) PCR product displays successful amplification of Syn1 (469bp) and EEF1A1 (1179bp) promoter sequences. Lane 1 contains 2-log ladder. (b) Based on the 2-log ladder, both bands appear to be about 1.1 kbp. Prepped for HiFi assembly. Lane 2 indicates SYN1Kozak-EGFP-T2A in EGFP-T2A-Cloning Site_pA fragment and Lane 3 indicates EF1Kozak-EGFP-T2A in EGFP-T2A-Cloning Site_pA fragment. (c) Illustration of AAVS1 plasmid backbone complete with insertion of HiFi assembled cell type specific promoter (Red) and EGFP (Green) sequences. Transfection of assembled cassettes into NPCs identified cell types based on EGFP expression within cells exclusively expressing the corresponding reporter gene promoter. An EcoRV restriction site (Yellow) was included for prospective CRISPR insert for cell type specific regulation.
Transformation into competent *E. coli*.

Chemically-competent Stbl3 *E. coli* cells were thawed on ice. 2ul of assembled HiFi product was added to these cells and incubated on ice for 30 minutes. The cells were then heat shocked for 30 seconds at 42°C and placed back on ice for 2 minutes. 950ul of SOC media was added to the tube at room temperature. The tube was incubated at 37°C, shaken for 1 hour. 100ul of transformed cell suspension was incubated overnight at 37°C on LB and LB+AMP agar plates for antibiotic selection and subsequent bacterial direct PCR for confirmation of successful transformation.

Transient transfection and differentiation of NPCs (day 0 - day 28)

*E. coli* cells were selected for successful transformation by bacterial direct PCR, and lysed. Assembled plasmid was harvested and then transfected with LipoD293 in confluent 24-well adherent plates of NPC cultures. LipoD293 transfection agent in DMEM was mixed together (per condition/promoter) with 500ng sgRNA A, 500ng sgRNA B, and 1ug AAVS1 assembled plasmid and incubated at 37°C for 15 minutes. Cell media was changed to NPCb + FGF and then treatment of 50ul LD293 (per condition) was administered to each designated well. Cultures were incubated at 37°C for ~8 hours. LD293 media was replaced with NPCb + FGF and cells were allowed to proliferate for ~48 hours before passaging to 48-well and 96-well plates for subsequent puromycin selection and differentiation via FGF withdrawal. In 3D cultures, NPCs were: transfected, allowed to grow confluent, suspended in NPCb + FGF, transferred to plates shaken at 90RPM, and differentiated once spheres were visually recognized.
Selection with puromycin

Puromycin antibiotic was used in concentrations of 2ng/ul, 1ng/ul, and 0.5ng/ul for the selection of successfully transfected neural progenitor cells. After treatment with puromycin, the cells were left to grow to confluence. Once cells were confluent, FGF withdrawal was performed to initiate cell differentiation.

Fluorescent imaging

Zeiss Axio ObserverZ1 inverted fluorescence microscope with digital camera aided in the visualization of transfected cells. After differentiation was initiated, cells were imaged every 3-5 days, beginning with day 0, for at least 28 days.

Drug Treatments and Cell Viability

Treatments were administered to cells according to the plate layout in Table 5. Resazurin/Resorufin assay was performed to determine cell viability. Drug treatments were prepared as four serial dilutions by a dilution factor of 1/3. To prepare concentration (A), 3ul of stock concentration was diluted into 500ul NPCb + 0.1% DMSO. Concentrations (B), (C), (D) were prepared by serial dilution of 200ul concentration (A) into 400ul NPCb (Table 2). On day 28 of differentiation, cells were replated and treated in triplicate for each concentration per condition. After 48 hours media plus drug treatments were removed and replaced with NPCb. Resazurin was prepared at 12.56mg/ml in HBSS and further diluted 1/5 in 10ml HBSS. Of the Resazurin dilution, 37.5ul was added to 150ul NPCb. Plates were incubated for 2 hours at 37°C and read on a SpectraMax i3 (Molecular Devices) on Softmax pro 6.4 software (208461) at λ 562nm.
Table 5. Two 96-well plates containing ~50,000 cells/well were loaded with drug treatments and controls according to this plate layout. Color coded for drug concentrations. Serial dilutions were performed and cells were treated in triplicate.

<table>
<thead>
<tr>
<th></th>
<th>Concentration A</th>
<th>Concentration B</th>
<th>Concentration C</th>
<th>Concentration D</th>
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<tr>
<td></td>
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<tr>
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<td>HBSS + DMSO</td>
<td>HBSS + DMSO</td>
<td>HBSS + DMSO</td>
<td>HBSS + DMSO</td>
</tr>
</tbody>
</table>

Serial dilutions were performed and controls were included.
Western blots

Extracted protein samples from drug treated cells were loaded at 0.4ug/ul on 4% Bis-Tris Midi Gels (NuPAGE, Ref. WG1403BX10); run at 200V for 40 minutes in MES-SDS (NuPAGE) running buffer; transferred to membrane using tris-glycine-20% methanol and kept there for 2 hours at 40V. Protein samples were standardized to a BCA assay. Membranes were blocked in milk for 45 minutes, washed, and incubated overnight in primary antibody: 1-2,000 (Sigma, Polyclonal Rabbit, anti-tau) or 1-25,000 (Millipore, MAB 1501 Monoclonal mouse, anti-actin) in 5% milk/TBST. Membranes were washed with TBS in 0.5% TWEEN20 and incubated with LC3/Tau secondary antibody (Vector Labs, goat anti-rabbit HRP PI-1000) or Actin secondary antibody (Vector Labs, horse anti-mouse HRP PI-2000). Western blot images (Figure. 6) were visualized on a C-DiGit blot scanner (Li-Cor, 3600).
Figure 6. Western blot displays Total tau, Actin, LC3-I, and LC3-II protein accumulation within control (Top) CV4a or diseased (Bottom) CV17q cells. Lane 1 represents chemiluminescent protein MW standards. Lanes 2-12 represent individual drug treatments with conditioned protein accumulation. Concentrations “B” and “D” were selected for each treatment based on a resazurin cell viability assay. Measurements of band pixel density were performed in ImageJ. One measurement was taken to include all five bands for total tau quantification and five separate measurements were taken for individually observed bands.
Gene Expression Quantification and Statistical Analysis

Band length of cDNA samples was determined by gel electrophoresis and pixel density was analyzed in ImageJ analysis software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA). A frame with a set area of 0.50 (Figure 7) was used to measure and quantify pixel density. Background measurements were averaged and subtracted from each measurement. Background subtracted measurements were normalized to constitutive housekeeping genes (GAPDH, TBP, RPL27, EEF1A1). Collected data was organized in Excel and statistically analyzed in SPSS. A heat map was produced to describe the entire panel of genes as compared to CV4a controls (Day 0 NPCs). Individual sample t-test was performed on pixilation data to compare levels of MAFT gene expression between cell lines.
Figure 7. Example of analyzed gel for gene expression profile. (TOP) Representative image of 2% gel after being loaded with PCR amplified cDNA and targeted primer sets. (BOTTOM) Pixel density was measured inside a fixed area represented by the numbered yellow boxes. Gene expression was determined to be up or down regulated as compared to normalized quantification to housekeeping gene(s).
Protein Quantification and Statistical Analysis

Protein levels were analyzed in ImageJ analysis software. A frame with a set area (Figure 8) was used to measure and quantify pixel density of individual bands per lane. Background measurements were averaged and subtracted from each measurement. Background subtracted measurements were normalized to constitutive actin as a control. Data was statistically analyzed in Excel or SPSS. First, an individual sample t-test was performed to compare baseline vehicle controls between CV4a and CV17q cell lines. One-way ANOVA was performed to compare protein levels between vehicle and selected drug concentrations (B, D) within the CV4a or CV17q cell line. Any significant changes in protein levels were tested for homogeneity of variance. Protein data that were found to have equal variance were further analyzed by Bonferroni correction, or data that were found not to have equal variance were further analyzed by Dunnett’s T3 test.

Figure 8. Example of analyzed gel for protein expression after drug treatments. Pixel density was measured inside a fixed area represented by the numbered yellow boxes. Protein levels were determined to be up or down regulated as compared to normalized quantification of actin.
RESULTS

Vehicle Baseline Control between Cell Lines

An individual sample t-test was first performed in order to compare the means of CV4a vs CV17q independent vehicles (NPCb + DMSO) between cell lines (Figure 9). This provided statistical evidence, assuming the equality of variance, that these: LC3-II/LC3-I (p>0.001), MAPT5 (p=0.043), and total tau (p=0.041) associated population means were significantly different.

![Figure 9](image_url) **Figure 9.** Baseline vehicle graph normalized to actin, or LC3-II/LC3-I ratio comparing CV4a to CV17q in normal media conditions. Boxes outline significance. There was a decreased of LC3-II/LC3-I ratio in CV17q (p<0.001), MAPT5 was increased in CV17q (p=0.043), and there was more total tau in CV17q (p=0.041).

Cell Viability per Drug Treatment

One-way ANOVA was performed to compare vehicle with four concentrations within the CV4a (Figure 10) or CV17q (Figure 11) cell line. Any significant changes in cell viability were tested for homogeneity of variance and further analyzed by Dunnett’s T3, or Bonferroni corrected. Within the CV4a line, Rapamycin, Torin, MG-132,
Simvastatin, Nystatin, FCCP, and PUaD measurements were found to have equal variance and therefore, Bonferroni corrected. Data that were found to not have equal variance, within the CV4a line, (SCD1, SPAUTIN1, BAFA1, Chloroquine, Vorinostat) were analyzed using Dunnett’s T3 test. Within the CV17q line, only Chloroquine measurements were found to have equal variance and subsequently Bonferroni corrected. Data that were found to not have equal variance (Rapamycin, Torin1, SCD1, SPAUTIN1, BAFA1, MG-132, Simvastatin, Nystatin, FCCP, PuAD, Vorinostat) were analyzed using Dunnett’s T3 test.

Within the CV4a line (Figure 10):

Rapamycin caused a decrease in LC3-II/LC3-I ratio by concentration D when compared to control (assumed equal variance; p=0.009). Torin1 induced a decrease of LC3-II/LC3-I by concentration D when compared to control (not assumed equal variance; p>0.001). Spautin1 had no significant changes in any protein levels by any concentration. BafA1 showed a significant increase in LC3-II/LC3-I ratio by concentration B (not assumed equal variance; p=0.017). Chloroquine exhibited increase of LC3-II/LC3-I ratio by concentration B (equal variance assumed; p=0.006). MG-132 displayed a significant increase in LC3-II/Actin by concentration B (equal variance not assumed; p=0.018), as well as LC3-II/LC3-I ratio increase by concentration B (p=0.003) but had a significant decrease by concentration D (p=0.030) (equal variance assumed), and an increase in MAPT4 by concentration B (equal variance assumed; p=0.019). Simvastatin showed a decrease in LC3-II/LC3-I by concentration D (not assumed equal variance; p=0.035). Nystatin caused a significant decrease in LC3-II/LC3-I by
concentration D (not assumed equal variance; p=0.001). PuAD decreased the LC3-II/LC3-I ratio by both concentrations B and D (assumed equal variance; p=0.031; p=0.004). PuAD also exhibited increases in MAPT4 by concentration B (assumed; p=0.004), MAPT5 by concentrations B and D (assumed; p=0.001; p=0.004), and total tau accumulation by concentration B (assumed; p=0.015). Vorinostat showed a decrease in LC3-II/LC3-I by concentrations B and D (assumed; p=0.012; p=0.006). Vorinostat also demonstrated increase in MAPT2 by concentration B (assumed equal variance; p=0.032), MAPT3 by concentration B (assumed; p=0.017), MAPT5 by concentration B (not assumed equal variance; p=0.029), and total tau accumulation by both concentrations (assumed; p=0.009; p=0.028).

Within the CV17q line (Figure 11):

Rapamycin had no significant effect on protein levels by any concentration when compared to control. Torin1 caused a decrease in MAPT2 accumulation by concentration D (assumed equal variance; p=0.031). Spautin1 caused an increase in MAPT2 and MAPT3 accumulation by concentration D (assumed equal variance; p=0.018, p=0.047 respectively). BafA1 showed a significant increase in LC3-II/LC3-I ratio by concentration B (not assumed equal variance; p=0.029). BafA1 also caused a decrease in MAPT1 by concentration D (assumed; p=0.004), MAPT2 by concentration D (assumed equal variance; p=0.002), MAPT3 by concentration D (assumed; p=0.005), MAPT5 by both concentrations (not assumed equal variance; p=0.031; p=0.015), and total tau accumulation by concentration D (assumed equal variance; p=0.013). Chloroquine exhibited decreases in MAPT1 by concentration D (assumed; p=0.008), MAPT2 by
concentration D (not assumed equal; p=0.001), and MAPT3 by concentration D 
(assumed; p=0.041). MG-132 had a significant decrease in MAPT1 by concentration D 
(assumed; p>0.001), as well as an increase in MAPT2 and MAPT4 by concentration B 
(assumed; p=0.002; p=0.001). Simvastatin showed a decrease in MAPT1 by 
concentration D (assumed; p=0.001) and MAPT2 by concentration D (assumed equal 
variance; p=0.045). Interestingly, Simvastatin exhibited a decrease in LC3-I protein when 
compared to control in concentration D (assumed; p=0.041). Nystatin displayed a 
significant increase in MAPT1, MAPT2, and MAPT3 by concentration B (assumed; 
p=0.001, p=0.007, p=0.004). PuAD exhibited increases in MAPT1 by concentrations B 
and D (assumed; p<0.001; p=0.005), MAPT2 by concentrations B and D (assumed equal; 
p<0.001; p=0.007), MAPT3 by concentration B only (assumed; p<0.001), MAPT4 by 
concentrations B and D (assumed; 0.000; p=0.003), MAPT5 by concentration B only 
(assumed; p=0.008), and total tau by both concentrations B and D (assumed equal 
variance; p<0.001; p=0.022). Vorinostat displayed an increase in MAPT1 by 
concentrations B and D (assumed equal; p<0.001; p<0.001), MAPT2 by concentrations B 
and D (assumed equal; p<0.001; p<0.001), MAPT3 by concentrations B and D (assumed 
equal; p<0.001; p<0.001), MAPT4 by concentrations B and D (assumed equal; p<0.001; 
p<0.001), MAPT5 by concentrations B and D (assumed equal; p<0.001; p=0.001), and 
total tau accumulation by concentrations B and D (assumed equal variance; p<0.001; 
p<0.001).

To validate drug efficacy, treatments were first tested for significant correlation to 
cell viability (Figure 10; Figure 11). Cell viability was measured via metabolism of
resazurin into resorufin in four different concentrations of previously described drug treatments (Table 2; Table 4) within two cell lines (CV4a, CV17q). Plates were incubated with resazurin and read for the absorbance of resorufin at $\lambda$ 562nm. Cell viability results, as well as protein accumulation in cultured iPSC derived neural cells vary with each treatment and are summarized in graphs (Figures 12-23) below.

![Figure 10. Cell viability by resazurin in the CV4a cell line. Each drug was used in four concentrations as A, B, C, or D. NPCb + 0.1% DMSO was the control. Rapamycin, Torin1, MG-132, Simvastatin, Nystatin, FCCP, and PuAD measurements were normally distributed and further analyzed using a Bonferroni correction test. Data found not to be normally distributed (SCD1, Spautin1, BafA1, Chloroquine, Vorinostat) were analyzed using the Dunnett’s T3 test. Rapamycin showed significant change in concentration C; Torin1 showed significant changes in concentrations B,C, and a trend for concentration A; SCD1 showed significant changes in concentrations A,B,C,D; Spautin1 showed significant changes in concentrations B,C, and a trend in concentration A; BafA1 showed significant changes in concentrations A,B,C,D; Chloroquine showed significant change in concentrations C,D; MG-132 showed significant changes in concentrations A,B,C; Simvastatin showed no significant changes from control; Nystatin showed no significant change from control; FCCP showed a trend in concentration A (p=0.057); PuAD showed significant changes in concentrations A,B; Vorinostat showed significant changes in concentrations A,B,C,D.](image-url)
Figure 11. Cell viability by resazurin in the CV17q cell line. Each drug was used in four concentrations as A, B, C, or D. NPCb + 0.1% DMSO was the control. Chloroquine measurements were normally distributed and further analyzed using a Dunnett’s T3 test. Data found not to be normally distributed (Rapamycin, Torin1, SCD1, Spautin1, BafA1, MG-132, Simvastatin, Nystatin, FCCP, PuAD, Vorinostat) were analyzed using the Bonferroni correction test. Rapamycin showed no significant change from control; Torin1 showed significant changes in concentrations A, B, C; SCD1 showed significant changes in concentrations A, B, C; Spautin1 showed significant changes in concentrations A, B, C; BafA1 showed significant changes in concentrations A, B, C, D; Chloroquine showed no significant change from control; MG-132 showed significant changes in concentrations A, B, C; Simvastatin showed significant changes in concentration A and a strong trend in concentration B (p=0.058); Nystatin showed no significant change from control; FCCP showed significant changes in concentrations A, C; PuAD showed significant changes in concentrations A, B, C; Vorinostat showed significant changes in concentrations A, B, C, D.
Protein Accumulation Varies with Condition and Cell Line

One-way ANOVA was performed to compare vehicle with selected concentrations (B, D) within the CV4a or CV17q cell lines. Any reported changes in protein level were tested for homogeneity of variance and further analyzed by Dunnett’s T3, or Bonferroni corrected.

Rapamycin

In four concentrations (A=5.48uM, B=1.83uM, C=0.61uM, D=0.20uM) (Figure 12) CV4a cells showed significant cell viability decrease, assessed by resazurin, in 0.61uM concentration, when normalized to control (Figure 12a). Western blot analysis revealed a decrease in LC3-II/LC3-I ratio after treatment with 0.20uM (Figure 12b). Treating CV17q cells with Rapamycin (Figure 12) showed no significant changes in cell viability by resazurin for any conditions (Figure 12a). Western blot results showed no significant changes in LC3-I or LC3-II protein accumulation (Figure 12b). Individual tau and total tau analyses (Figure 12c) exhibited no significant change in tau accumulation within either CV4a or CV17q cell lines.
Figure 12. Effects of treating cell lines with Rapamycin in four concentrations. (a) Treatment with rapamycin shows cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment) cell lines. There was a decrease in CV4a cell viability by 0.61uM (p=0.007). (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or LC3-II/LC3-I ratio. Concentration D affects LC3-II/LC3-I accumulation in CV4a (p=0.009). (c) Individual tau band and total tau analyses show no effect on tau protein levels. Graph, mean ± SEM, n=9 Treatment, n=7 Control.

In four concentrations (A=2.04uM, B=0.68uM, C=0.23uM, D=0.08uM) (Figure 13) CV4a showed a decrease in cell viability by 0.68uM, and 0.23uM normalized to control (Figure 13a). Western blot results showed that 0.68uM has a decreasing effect on LC3-II/LC3-I ratio (Figure 13b). Effects of treating CV17q cells with Torin1 (Figure 13) showed a decrease in cell viability by 2.04uM, 0.68uM, and 0.23uM (Figure 13a). Western blot results show no change in LC3 levels (Figure 13b). Individual tau and total tau analysis (Figure 13c) exhibited a decrease of MAPT2 accumulation within CV17q.
Figure 13. Effects of treating cell lines with Torin1 in four concentrations. (a) Treatment with Torin1 showed cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was a change in CV4a cell viability by 0.68uM (p=0.014), 0.23uM (P=0.021) and a change in CV17q cell viability by 2.04uM (p=0.008), 0.68uM (p=0.001), and 0.23uM (p=0.034). (b) Western blot results showed LC3-I (left) and LC3-II (right) accumulation normalized to actin or ratio of LC3-II/LC3-I. 0.08uM affects LC3-II/LC3-I ratio in CV4a (p<0.001). (c) Individual tau band and total tau analyses showed a decreasing effect on MAPT2 levels in CV17q cells (p=0.031). Graph, mean ± SEM, n=9 Treatment, n=72 Control.

SCD1

In four concentrations (A=20.8uM, B=7.0uM, C=2.36uM, D=0.79uM) (Figure 14) CV4a showed significant decrease in cell viability by resazurin for 20.8uM, 7.0uM, 2.36uM, and 0.79uM normalized to control. Effects of treating CV17q cells with SCD1 showed significant changes in cell viability (Figure 14) by resazurin in 20.8uM, 7.0uM, and 2.36uM. Decreased cell viability in all concentrations with the exception of 0.79uM in CV17q cells indicates a negative effect of SCD1 on cell viability.
Spautin1

In four concentrations (A=42.4uM, B=14.3uM, C=4.85, D=1.64uM) (Figure 15) CV4a showed a decrease in cell viability by resazurin in 14.3uM normalized to control (Figure 15a). Western blot showed no changes to protein accumulation (Figure 15b).

Effects of treating CV17q cells with Spautin1 (Figure 15) showed a decrease in cell viability by 14.3uM, and 4.85uM (Figure 15a). Western blot showed no significant changes in LC3 protein levels (Figure 15b). Individual tau and total tau analyses (Figure 15c) exhibited a change in MAPT2 and MAPT3 protein levels at 1.64uM within the CV17q cell line.
**Figure 15.** Effects of treating cell lines with Spautin1 in four concentrations. (a) Treatment with Spautin1 show cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was a change in CV4a cell viability by 14.3uM (p<0.001). There was a decrease in CV17q cell viability by 14.3uM (p<0.001), and 4.85uM (p<0.001). (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or LC3-II/LC3-I ratio. There were no changes in either cell line at either concentration. (c) Individual tau band and total tau analyses show 1.64uM has an effect on MAPT2 (p=0.018), and MAPT3 (p=0.047) protein levels in CV17q cells. Graph, mean ± SEM, n=9 Treatment, n=72 Control.

**Bafilomycin A1**

In four concentrations (A=216nM, B=73.44nM, C=24.97nM, D=8349nM)

(Figure 16) CV4a showed significant decrease in cell viability by resazurin for all concentrations when normalized to control (Figure 16a). Western blot showed that there was no significant change in LC3-I (Figure 16b) and a significant decrease in LC3-II/LC3-I ratio by 73.44nM (Figure 16b). Effects of treating CV17q cells with BAFA1 (Figure 16) showed significant decrease in cell viability by all concentrations when compared to control (Figure 16a). Western blot showed no change in LC3-I and significant increase in LC3-II/LC3-I ratio by 73.44nM (Figure 16b). Individual tau and total tau analyses (Figure 16c) exhibited a decrease in MAPT1, MAPT2, MAPT3,
MAPT5, and total tau protein within the CV17q line at 8.49nM and an increase in MAPT5 by 73.44nM.

**Figure 16.** Effects of treating cell lines with BAF1 in four concentrations. (a) Treatment with BafA1 showed cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was a decrease in CV4a cell viability by resazurin at 216nM (p<0.001), 73.44nM (p<0.001), 24.97nm (p<0.001), and 8.49nM (p=0.004). There was a decrease in CV17q cell viability by all four concentrations compared to control (p=0.009), (p<0.001), (p<0.001) (p=0.001). (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin and LC3-II/LC3-I. 73.44nM affects LC3-II/LC3-I ratio in CV4a and CV17q lines (p=0.017), (p=0.029) (c) Individual tau band and total tau analyses show a decrease in MAPT1 (p=0.004), MAPT2 (p=0.002), MAPT3 (p=0.005), MAPT5 (p=0.015), and total tau protein (p=0.013) within the CV17q line at 8.49nM and an increase in MAPT5 at 73.44nM (p=0.031). Graph, mean ± SEM, n=9 Treatment, n=7 Control.

**Chloroquine**

In four concentrations (A=220ug/mL, B=75.17ug/mL, C=25.68ug/mL, D=8.77ug/mL) **(Figure 17)** CV4a showed a decrease in cell viability by resazurin for 25.68ug/ml, and 8.77ug/ml normalized to control **(Figure 17a).** Western blot showed no significant change in LC3-I levels. There was an increase of LC3-II/LC3-I ratio after treating with 75.17ug/mL **(Figure 17b).** Effects of treating CV17q cells with
Chloroquine (Figure 17) showed no significant change in cell viability by resazurin for any concentration (Figure 17a). Western blot showed no change in LC3-I or LC3-II (Figure 17b). Individual tau and total tau analysis (Figure 17c) exhibited a decrease in MAPT1, MAPT2, and MAPT3 after treatment with 8.77µg/mL within CV17q.

**Figure 17.** Effects of treating cell lines with Chloroquine in four concentrations. (a) Treatment with Chloroquine show fold changes in cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was a decrease in CV4a cell viability by 25.68µg/ml (p<0.001), and 8.77µg/mL (p=0.006). There were no reported changes in CV17q cell viability by any of the four concentrations as compared to control. (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or ratio of LC3-II/LC3-I. 75.17µg/mL showed an increase in LC3-II/LC3-I ratio in CV4a (p=0.006). (c) Individual tau band and total tau analyses show an increase within the CV17q line of MAPT1 (p=0.008), MAPT2 (0.001), and MAPT3 (p=0.041) after treatment with 8.77µg/mL and no change in CV4a. Graph, mean ± SEM, n=9 Treatment, n=72 Control.

MG-132

In four concentrations (A=22.4uM, B=7.69uM, C=2.64uM, D=0.91uM) (Figure 18) CV4a and CV17q displayed a decrease in cell viability by resazurin for 22.4uM, 7.69uM, and 2.64uM normalized to control (Figure 18a). Western blot showed no significant changes in LC3-I protein in either cell line (Figure 18b) 7.69µM and 0.91µM
show a significant effect on LC3-II/LC3-I within CV4a cells. Individual tau and total tau analyses normalized to actin (Figure 18c) exhibited a significant increase in MAPT4 at 7.69uM in CV4a. Tau analysis also exhibited an increase in MAPT1, MAPT2, and MAPT4 in CV17q. The effect of MG-132 on cell viability may be caused by higher concentrations indicated by no change at 0.91uM only.
Figure 18. Effects of treating cell lines with MG-132 in four concentrations. (a) Treatment with MG-132 show fold changes in cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was a decrease in CV4a cell viability by 22.4uM (p<0.001), 7.69uM (p<0.001), and 2.64uM (p=0.002). There was a decrease in CV17q cell viability by 22.4uM (p<0.001), 7.69uM (p<0.001), and 2.64uM (p<0.001). (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or ratio of LC3-II/LC3-I. 7.69uM (p=0.003) and 0.91uM (p=0.030) showed an increase in LC3-II/LC3-I accumulation in CV4a only. (c) Individual tau band and total tau analyses normalized to actin show an increase in MAPT4 (p=0.019) after treatment with 7.69uM in CV4a. Tau analysis also exhibited a decrease within the CV17q line, in MAPT1 (p<0.001) by 0.91uM, MAPT2 (p=0.002) by 7.69uM, and MAPT4 (p=0.001) in 7.69uM. Graph, mean ± SEM, n=9 Treatment, n=72 Control.

Simvastatin

In four concentrations (A=22.8uM, B=7.87uM, C=2.71uM, D=0.94uM) (Figure 19) CV4a showed no significant changes in cell viability by resazurin in any condition (Figure 19a). Within the CV4a cell line, Western blot showed a significant decrease in LC3-II/LC3-I after treatment with 0.94uM (Figure 19b). Effects of treating CV17q cells with Simvastatin (Figure 19) showed a decrease in cell viability by resazurin in 22.8uM (Figure 19a). Western blot showed significant decrease in LC3-I protein accumulation at 0.94uM when normalized to actin (Figure 19b). Individual tau and total tau analyses
normalized to actin (Figure 19c) exhibited no change in tau levels within the CV4a line at either concentration when compared to control. Tau analysis exhibited a significant decrease in MAPT1, and MAPT2, within CV17q at 0.94uM.

Figure 19. Effects of treating cell lines with Simvastatin in four concentrations. (a) Treatment with Simvastatin show fold changes in cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was no change in CV4a cell viability by any concentration and treatment showed a decrease in CV17q cell viability at 22.8uM (p=0.005). (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or ratio of LC3-II/LC3-I. 0.94uM seemed to have a decreasing effect on LC3-II/LC3-I ratio (p=0.035) in CV4a. Cells treated with 0.94uM also displayed a decrease of LC3-I (p=0.041) in CV17q. (c) Individual tau band and total tau analyses normalized to actin show that 0.94uM has a decreasing effect on MAPT1 (p=0.001), and MAPT2 (p=0.001) in CV17q cells. Graph, mean ± SEM, n=9 Treatment, n=72 Control.

Nystatin

In four concentrations (A=11.6ug/mL, B=4.02ug/mL, C=1.39ug/mL, D=0.48ug/mL) (Figure 20) CV4a and CV17q showed no significant change in cell viability by resazurin in any concentration (Figure 20a). Within the CV4a cell line, Western blot showed a significant reduction in LC3-II/LC3-I ratio after treatment with 0.48ug/ml (Figure 20b). Within the CV17q cell line, Western blot presented no changes
to LC3-I or LC3-II (Figure 20b). Individual tau and total tau analyses normalized to actin (Figure 20c) exhibited an increase in MAPT1, MAPT2, and MAPT4 tau levels within the CV17q line at 4.02µg/mL when compared to control.

**Figure 20.** Effects of treating cell lines with Nystatin in four concentrations. (a) Treatment with Nystatin show fold changes in cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was no change in either CV4a or CV17q cell viability by any concentration. (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or ratio of LC3-II/LC3-I. There was a decrease in LC3-II/LC3-I ratio (p=0.001) in CV4a by 0.48µg/ml. (c) Individual tau band and total tau analyses normalized to actin show an increase in MAPT1 (p=0.001), MAPT2 (p=0.007), and MAPT4 (p=0.004) within the CV17q cell line after treatment with 4.02µg/ml.

**FCCP**

In four concentrations (A=47.2µM, B=16.44µM, C=5.73µM, D=1.99µM) (Figure 21) showed no change in cell viability by resazurin in CV4a. Effects of treating CV17q cells with FCCP (Figure 21) showed significant decrease in cell viability by resazurin for 47.2µM and 5.73µM when compared to control.
Figure 21. Effects of treating cell lines with FCCP in four concentrations. Treatment with FCCP show cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was only a decrease in CV17q cell viability by 47.2uM (p=0.004), and 5.73uM (p=0.001).

PuAD

In four concentrations (A=1200nM, B=420nM, C=147nM, D=51.45nM) (Figure 22) CV4a showed significant decrease in cell viability by resazurin for 1200nM and 420nM (Figure 22a). Western blot showed a significant decrease in LC3-II/LC3-I ratio by 51.45nM (Figure 22b). Effects of treating CV17q cells with PuAD (Figure 22) showed significant decrease in cell viability by resazurin for 1200nM, 420nM, and 147nM (Figure 22a). Individual tau and total tau analyses normalized to actin (Figure 22c) exhibited an increase in MAPT4, MAPT5, and total tau within the CV4a line. Tau analysis also exhibited significant increases in MAPT1, MAPT2, MAPT3, MAPT4, MAPT5, and total tau within the CV17q line.
Figure 22. Effects of treating cell lines with PuAD in four concentrations. (a) Treatment with PuAD shows fold changes in cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was a decrease to cell viability in CV4a by 1200nM (p<0.001), and 420nM (p=0.005). There was a decrease to CV17q cell viability by 1200nM (p<0.001), 420nM (p<0.001), and 147nM (p=0.029). (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or ratio of LC3-II/LC3-I. There was a decrease in LC3-II/LC3-I in CV4a by 420nM (p=0.031) and 51.45nM (p=0.004). (c) Individual tau band and total tau analysis normalized to actin show an increase in MAPT4 at 420nM (p=0.004), MAPT5 at 420nM (p=0.001) and 51.45nM (p=0.004) within the CV4a cell line. Total tau levels seem to increase (p=0.015) by 420nM. Tau analysis indicates there were also increases in MAPT1 by 420nM (p<0.001) and 51.45nM (p=0.005), MAPT2 by 420nM (p<0.001) and 51.45nM (p=0.007), MAPT3 by 420nM only (p=0.001), MAPT4 by 420nM (p<0.001) and 51.45nM (p=0.003), MAPT5 by 420nM (p=0.008), and total tau accumulation by 420nM (p<0.001) and 51.45nM (p=0.022) within the CV17q cell line.

Vorinostat

In four concentrations (A=122uM, B=42.90uM, C=15.09uM, D=5.31uM) (Figure 23) CV4a showed significantly decreased cell viability by resazurin for all four concentrations in both CV4a and CV17q cell lines (Figure 23a). Western blot displayed a decrease in LC3-II/LC3-I ratio by 42.90uM and 5.31uM within the CV4a line. Within the CV17q line, there was no change in LC3-I or LC3-II after treatments (Figure 23b). Individual tau and total tau analyses normalized to actin (Figure 23c) exhibited an
increase in MAPT2, MAPT3, MAPT5, and total tau within CV4a. Tau analysis also exhibited an increase in MAPT1, MAPT2, MAPT3, MAPT4, MAPT5, and total tau within CV17q.

**Figure 23.** Effects of treating cell lines with Vorinostat in four concentrations. (a) Treatment with Vorinostat show fold changes in cell viability by resazurin in CV4a (n=6 Control, n=3 Treatment) and CV17q (n=10 Control, n=5 Treatment). There was a decrease in cell viability by resazurin in both CV4a and CV17q cell viability by all concentrations (p<0.001). (b) Western blot results show LC3-I (left) and LC3-II (right) accumulation normalized to actin or ratio of LC3-II/LC3-I. Western blot showed a decrease in LC3-II/LC3-I ratio by 42.90uM and 5.31uM (p=0.012; p=0.006) within the CV4a line. Individual tau and total tau analyses normalized to actin exhibited an increase in MAPT2 by 42.90uM (p=0.012), MAPT3 by 42.90uM (p=0.017), MAPT5 by 42.90uM (p=0.029), and total tau by 42.90uM (p=0.009) and 5.31uM (p=0.028) within CV4a. Tau analysis also exhibited an increase in MAPT1 by both concentrations (p<0.001; p<0.001), MAPT2 by both concentrations (p<0.001, p<0.001), MAPT3 by both concentrations (p<0.001, p<0.001), MAPT4 by both concentrations (p<0.001, p<0.001), MAPT5 by both concentrations (p<0.001, p<0.001), and total tau by both concentrations (p<0.001, p<0.001) within CV17q.
Gene Expression Profiles in 2D vs 3-D Formats

Gene expression was analyzed by RT-PCR and results are displayed in Figure 24. Hierarchical clustering shows the similarities and differences based on the expression of each gene as increasing or decreasing when compared to CV4a control, Day 0 NPCs. Neural progenitor cells (Day 0) show low expression of neuronal, or oligodendrocytic genes in either CV4a or CV17q cell lines. Low levels of some astrocytic genes (Ptx3, Tcfcp2l1, Dmp1) can be seen being expressed in CV17q NPCs (Day 0). Adherent cells at day 28 show an increase in neuronal and astrocytic gene expression within both cell lines indicating mixed populations of cells and consistent with expected results. Neural sphere cultures at day 28 show some expression of only astrocytic genes within the CV4a line. Results indicate an upregulation of only neuronal genes, as well as increased MAPT expression in CV17q as compared to control NPCs at Day 0. One observation worth noting is that 4R MAPT was highly expressed and upregulated in all but one sample.

Because I was interested in tau protein expression, I looked specifically at MAPT protein variants. Leven’s test for equal variance found no significant differences in any group. Individual sample t-tests for equality of means determined MAPT3R in CV4a 3D Day 28 (n=4) to be significantly different (p=0.016) from CV17q 3D Day28 (n=4).
Figure 24. Heat map displays gene expression analysis by hierarchical clustering and based on cell line (CV4a/CV17q) and cell type/format. (Left) NPC Day 0 cultures displayed low levels of all selected genes. (Middle) Adherent Day 28 cultures showed signs of containing all cell types based on gene expression. (Right) 3-D Day 28 cultures showed evidence of variable cell types based on gene expression between cell lines and between replicates CV4a. White/grey/black gradient shows intensity of gene regulation as compared to CV4a NPC control. Each box has labeled intensity from 0-1. White boxes show downregulation, black boxes show upregulation, and grey shows no change in gene expression.
Transiently Transfected Cells Display Signs of Successful Plasmid Integration.

Preliminary data in HEK293T cells (Figure 25) show high levels of GFP expression based on reporter gene promoters. HEK293t cells normally express EEF1A1 gene and as expected EGFP expression was present after transient transfection of the EEF1A1-GFP plasmid cassette (Figure 25A). EGFP expression was also present after transfection with the EEF1A1-CRISPR plasmid (Figure 25B). Low levels of EGFP was present after transfection with the SYN1-GFP cassette (Figure 25C). Interestingly, HEK293t cells do not naturally express the SYN1 gene which may indicate abnormal gene expression, autofluorescence, or integration of EGFP via CRISPR.

Fluorescence microscopy reveals EGFP expression within specific cell types (Figure 26). Transfection rate/successful transfections could not be confirmed; however, specific cell types may be distinguished/distinct from each other based on morphology. Results of EGFP expression confirmed the presence of EGFP in most transfected cultures.
Figure 25. EGFP expression in HEK293t cells based on reporter gene(s). All panels show varying degrees of EGFP expression. EEF1A1 reporter was used in panels A and B, SYN1 reporter was used in panel C. A) EGFP is highly expressed in HEK293t cells assumed to be caused by cell expression and induction by EEF1A1 promoter. B) HEK293t cells treated with EEF1A1-CRISPR cassette containing EGFP. C) HEK293t cells express low levels of EGFP after transfected with SYN1-CRISPR cassette. EGFP expression assumed to be due to CRISPR integration, though the SYN1 promoter should only be expressed in mature neurons. D) Negative control; no plasmid added.
Figure 26. Fluorescent microscopy reveals EGFP expression in iPSC derived neural cells. Cells were transfected with plasmid cassettes containing EGFP sequences induced by a cell type specific reporter promoter. (TOP) Brightfield microscopy (MIDDLE) GFP filter only (BOTTOM) Overlay of mCHERRY, GFP and brightfield images. After transfection, cells were subsequently differentiated by FGF withdrawal. A) EEF1A1, nine days differentiated. B) GFAP, nine days differentiated. C) CNP, nine days differentiated. D) SYN1, eighteen days differentiated. E) PRNP, twenty-eight days differentiated.

EEF1A1 reporter designed to identify all cell types

Cells transfected with the EEF1A1 plasmid cassette express EGFP up to 24 hours after transfection. EGFP expression was monitored across a differentiation period of 28 days. EGFP expression was observed as early as 24 hours after transfection up to 21 days (Figure 27). Morphology of cells are visually consistent with NPCs at day 0 and immature development of other cells at day 9. Morphology of astrocytes expressing EGFP was observed at around day 9 and up to day 21. Neuronal cell morphology was observed at day 9 and day 21.
Figure 27. Neural cells after transfection with housekeeping EEF1A1-GFP plasmid cassette. Fluorescent microscopy reveals EGFP expression in iPSC derived neural cells. (TOP) Cells transfected with plasmid cassettes containing EGFP sequences induced by EEF1A1 reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows NPCs at day 0 of differentiation, 24 hours after transfection. Lane 2 shows nine days differentiated. Lane 3 shows 21 days differentiated. (BOTTOM) Enlarged image of differentiation day 21 displays cells expressing GFP. Red arrow shows possible neuronal cell type; red circle shows possible astrocyte cell types.
GFAP reporter designed to identify astrocytes

Cells transfected with the GFAP plasmid cassette express EGFP as soon as 9 days after differentiation. EGFP expression was monitored across a differentiation period of 28 days. EGFP expression was observed as early as 9 days after transfection up to 28 days (Figure 28). As expected, the morphology of cells is consistent with those of astrocytes only. EGFP expression was observed at around day 9 and up to day 28.
Figure 28. Neural cells after transfection with astrocytic GFAP-GFP plasmid cassette. Fluorescent microscopy reveals EGFP expression in iPSC derived neural cells. (TOP) Cells transfected with plasmid cassettes containing EGFP sequences induced by GFAP reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows cells at day 2 of differentiation. Lane 2 shows nine days differentiated. Lane 3 shows 21 days differentiated. Lane 4 shows 28 days differentiated. (BOTTOM) Enlarged image of differentiation day 9 exhibits cells expressing EGFP with a morphology resembling astrocytes.
**CNP reporter designed to identify oligodendrocytes**

Cells transfected with the CNP plasmid cassette express extremely low levels of EGFP. EGFP expression was monitored across a differentiation period of 28 days (Figure 29). Neither morphology of cells nor cell types containing EGFP can be determined or confirmed, but expression may be observed as early as 9 days after differentiation.
Figure 29. Neural cells after transfection with oligodendrocytic CNP-GFP plasmid cassette. Fluorescent microscopy reveals extremely low levels of EGFP expression in iPSC derived neural cells. (TOP) Cells transfected with plasmid cassettes containing EGFP sequences induced by CNP reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows cells at day 2 of differentiation. Lane 2 shows nine days differentiated. Lane 3 shows 18 days differentiated. (BOTTOM) Enlarged image of differentiation day 9 exhibits cells expressing EGFP. Cell types cannot be confirmed.
SYN1 reporter designed to identify neurons with synapses

Cells transfected with the SYN1 plasmid cassette express EGFP as soon as 9 days after differentiation. EGFP expression was monitored across a differentiation period of 28 days. EGFP expression was observed as early as 9 days after transfection up to 18 days after differentiation (Figure 30). As expected, the morphology of cells that contain EGFP is visually consistent with those of neuronal cells. EGFP expression was observed at around day 9 and up to day 28 (not shown).

Figure 30. Neural cells after transfection with Neuronal SYN1-GFP plasmid cassette. Fluorescent microscopy reveals relatively low levels of EGFP expression in iPSC derived neural cells. Cells transfected with plasmid cassettes containing EGFP sequences induced by SYN1 reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. Lane 1 shows cells at day 2 of differentiation. Lane 2 shows 9 days differentiated. Lane 3 shows 18 days differentiated. Enlarged images (indicated by red lines) exhibit cells expressing EGFP after 18 days of differentiation.
PRNP reporter designed to identify mature neurons

Cells transfected with the PRNP plasmid cassette express EGFP as soon as 9 days after differentiation. EGFP expression was monitored across a differentiation period of 34 days. EGFP expression was observed as early as 9 days after transfection up to 34 days after differentiation (Figure 31). Cells expressing EGFP are assumed to be expressing the cell type specific gene PRNP. As expected, the morphology of cells is visually consistent with those of neuronal cells. EGFP expression was observed at around day 9 and up to day 34.
Figure 31. Neural cells after transfection with neuronal PRNP-GFP plasmid cassette. Fluorescent microscopy reveals relatively high levels of EGFP expression in iPSC derived neural cells. Cells transfected with plasmid cassettes containing EGFP sequences induced by SYN1 reporter promoter. After transfection, cells were subsequently differentiated by FGF withdrawal. (TOP) Lane 1 shows cells at day 2 of differentiation. Lane 2 shows 9 days differentiated. Lane 3 shows 28 days differentiated. Lane 4 shows 34 days differentiated. (BOTTOM) Enlarged image exhibits cells expressing EGFP after 34 days of differentiation.
Cells transfected without a plasmid cassette displayed some nonspecific and unconfirmed EGFP expression. EGFP expression was monitored across a differentiation period of 28 days. Some EGFP expression was observed (Figure 32) but the morphology of cells may not be consistent with a specific cell type. It was not expected, but could there be some other mechanism of expressing EGFP?

**Figure 32.** Neural cells after transfection with no plasmid cassette. Fluorescent microscopy reveals some EGFP expression in iPSC derived neural cells. After “transfection”, cells were subsequently differentiated by FGF withdrawal. Lane 1, cells at day 21 of differentiation. Lane 2, 25 days differentiated. Lane 3, 28 days differentiated.
DISCUSSION

Cell Culture and Differentiation

Human iPSCs and induced neural cells have allowed insight into the phenotypes of different neurodegenerative diseases (Israel et al. 2012). However, across tauopathies, it is unclear (i) which MAPT transcript variants and isoforms are predominant within disease-susceptible cells, (ii) how different tau isoforms are post-translationally modified and cleared from these susceptible cells, and (iii) whether there are differences in clearance and survival mechanisms between affected cell types (e.g. neurons vs. astrocytes).

Upon differentiation in neuronal media following FGF withdrawal, adhered monolayer cultures commonly produce cultures where restricted spatial factors establish connectome expansion (D’Aiuto et al. 2018). When NPCs are differentiated in either adhered or 3-D format, they are signaled to become one of the cell types of the brain. An important outcome of this project is that 3-D cultures did not necessarily differentiate to contain increased expression of astrocytic genes and/or oligodendrocytic genes (e.g. GFAP, CNP, etc.) in addition to neuronal gene expression; indicating restricted cell type differentiation. Monolayer cultures however, were expected to have a higher expression of neuronal genes with low expression of astrocytic/oligodendrocytic genes, due to the >90% neurons present in culture yet results indicate a constant expression within these cultures. Even though >90% of adherent cells become neurons, non-neuronal cell types are critical in the roles of neural support, maintenance, and differentiation (Rodriguez et
al. 2009, Weber and Barros 2015). Though results did not support the hypothesis that 3-D neural cultures are more analogous to in vivo brain cells; the goal to produce mixed populations of neuronal and non-neuronal/glial cell types was achieved.

Due to difficulty in developing homogenous cell populations, the majority of recent studies attempt to recapitulate in vivo cell types using homogenized brain tissue (Tagliafierro et al. 2016). In this study, monolayer cultures representing homogenous cell populations were compared to 3-D cultures that represent brain homogenates. The 3-D were more prone to microbial contamination during collection, and an inadequate number of cells for differentiation was acquired. These factors also affected the quality of obtainable RNA. In addition to clearing samples of genomic DNA and protein contamination, using a more robust RNA extraction method may achieve increased greater quantities of purified RNA samples. It is critical that precautions are taken when collecting cells in order to minimize contact with contaminated surfaces and laboratory equipment. The total number of initial cells should be increased in 3-D cultures to ensure a sufficient quantity of cells and subsequently RNA extracts. Geometry and composition have been seen to influence genes’ expression, and some genes promoting cell proliferation are repressed in 3-D culture (Edmondson et al. 2014). The original seeding density was determined by previous lab protocols based on proliferation rates of adherent cultures therefore proliferation, growth and development of 3-D cultures should be thoroughly examined before revisiting this experiment.
Protein Accumulation

Preliminary studies on the amplification of the 17q chromosome have indicated an increase in MAPT gene products resulting from a third copy of the MAPT gene (Dizon 2016). This work has established an increased expression of tau transcripts in iPSC lines as a possible diseased neural model. Due to copy number variation on chromosome 17, cell cultures of mixed neuronal/glia or purified neurons were characterized by RT-qPCR for MAPT 3R and 4R tau protein expression indicative of mixed tauopathies such as chronic traumatic encephalopathy, and Frontotemporal dementia and Parkinsonism linked to Chromosome 17; including evaluation of how total tau levels are influenced by this third copy of the MAPT gene. Western blot was used to measure protein levels in wildtype CV4a vs several experimental lines including CV17q. Tau levels were determined to be significantly higher in experimental cells, signifying either an increase in MAPT expression or an impairment of turnover processes. The Western blot was incubated with isoform specific tau antibodies and band sizes were found to be consistent with and assumed 3R2N and 3R1N tau isoforms. Based on the MSD pT231/total tau assay, it is likely that altered turnover is driving tau accumulation (Lotfy 2016). Based on these results, it appears that mixed populations of cells tend to accumulate more tau protein when compared to purified neuronal cultures. This may indicate that excess tau is produced, or accumulated, in non-neuronal or supportive glial cells. Immunocytochemistry on mixed vs purified cultures might determine whether or not neuronal cells exclusively yield a tau accumulation response in different cellular
environments. When cell viability is decreased, any subsequent reports of change at the protein level may be caused by a decrease in living cells within that culture or possible toxicity in the treatment(s). As an indicator of autophagosome formation, any observed drug effects on the LC3 protein accumulation is dependent on LC3-I conversion into LC3-II. When LC3-II protein levels increase, the cause may be that the drug is either an inhibitor of the ubiquitin proteasome system and activation of autophagy has been achieved, or that the autophagy pathway has been inhibited and the cells do not clear autophagosomes.

Autophagy and Ubiquitin Proteasome Degradative Pathways

Misfolded tau protein is degraded primarily by the autophagy pathway and may be alternatively degraded via ubiquitin proteasome (Lee et al. 2013). Drug treatment and molecular MAPT targeting may analyze tau degradation via autophagy and/or ubiquitin binding. In this study, a library of activators and inhibitors of autophagy and ubiquitin binding were screened (Table 1) and tau accumulation was directionally quantified by Western blot. My hypothesis was that activation of autophagy will stimulate the degradation of total tau protein whereas inhibition of the autophagy pathway results in accumulation of misfolded and/or native tau causing accelerated cell death in each cell type. It is likely that the autophagy and UPS pathways degrade distinct/different tau proteins, and so it is important to identify which tau proteins are accumulating and exclusive to inhibition of either pathway(s).
Drug Panel

Cell viability results indicate that an observed decrease at a treatment concentration that produced a significant change in cell metabolism by resazurin, regardless of cell line, may or may not implicate an effect of the drug. While there were different correlations between concentration and treatment (*Figure 11; Figure 12*) analysis of concentrations B and D were selected to keep consistency between treatments (*Table 2; Table 5*). Concentrations B and D were chosen because in most cases, concentration B showed there was at least some reduction in cell viability after treatment while concentration D frequently displayed the least amount of cell viability change. SCD1 and FCCP were excluded completely from protein analysis in the interest of time.

Inhibiting mTOR mimics starvation of the cell which in turn stimulates autophagy and signals homeostatic processes within the cell (Altomare and Khaled 2012). It was expected that treating cells with Rapamycin would either reduce protein levels by inducing autophagy or that protein levels would remain the same due to constitutive autophagy. The decrease as seen in LC3-II/LC3-I ratio by rapamycin was not consistent with other results. Rapamycin at 20uM increases LC3-II production indicating enhanced autophagosome formation (Lin *et al.* 2018). In the present study, we see a reduction of LC3-II/LC3-I by Rapamycin at 0.20uM within CV4a cells which could indicate normal autophagosome clearance. No change in protein accumulation could also indicate normal autophagy function, or that concentrations were too low to produce any significant effects.
Similar to the effects of Rapamycin, I expected that treating cells with Torin1 would reduce protein levels through autophagy induction or that protein levels would remain the same due to constitutive autophagy. Torin1 aggravates cytotoxicity (Huang et al. 2017), suggesting that mTOR inhibition may not be able to alleviate neurotoxicity. In the present study, the decrease seen in cell viability at concentrations higher than 0.08uM could be due to toxicity. As with the presence of LC3-II protein being an indicator of autophagosome formation, the reduction of LC3-II/LC3-I by Torin1 could indicate autophagosome clearance.

Spautin1, a small molecule inhibitor of ubiquitin-specific peptidase 10 and 13 affects autophagy by LC3-II inhibition (Liu et al. 2011). An observed decrease of cell viability by 14.3uM and 4.85uM in both cell lines are consistent with decreased cell survival (Schott et al. 2018) though this study showed no significant changes in LC3 accumulation.

It was hypothesized that inhibition of autophagy with BafA1 would render derived neural cells incapable of autophagosome formation which may be what so negatively affected cell viability in all concentrations. Increase of LC3-II/LC3-I in both CV4a and CV17q cell lines by 73.44nM support this hypothesis indicating impaired autophagy at this concentration. The decrease of MAPT1, MAPT2, MAPT3, MAPT5, and total tau observed in CV17q cells at 8.49nM could indicate an alternative mechanism by which the tau protein is cleared from the cells and CV17q may be more sensitive to tau degradation after treatment with BafA1. Another possibility could be because CV17q accumulates more tau protein.
Inhibition with chloroquine should increase tau protein, but findings suggest an autophagy independent, ubiquitin proteasome pathway and separate mechanism for tau degradation. Inhibition of lysosomal acidification with chloroquine disrupts autophagy function (Kimura et al. 2013). This may be the source of LC3-II/LC3-I increase in CV4a cells. The MAPT1, MAPT2, and MAPT3 decrease of tau proteins observed in CV17q cells at 8.77μg/mL indicate an alternative mechanism by which these tau isoforms (but not others) are cleared from the cells. CV17q may be more sensitive to tau degradation after treatment with Chloroquine, or because CV17q accumulates more tau protein it is much easier to see. The number of cells and/or total protein required to evaluate this must be increased in the CV4a cell line.

After treatment with MG-132, an increase of LC3-II within the CV4a cell line at 7.69μM and decrease by 0.91μM implicate higher concentrations of MG-132 in cell death/apoptosis or that higher concentrations reduce the degradation of ubiquitin conjugated proteins by which LC3-II accumulation occurs. The increase of MAPT4 in CV4a could be the effect of drug treatment, or the source of decreased cell viability. Interestingly, in CV17q, the decrease of MAPT1 by 0.91μM and increase of MAPT2 and MAPT4 by 7.69μM may suggest a separate mechanism by which MAPT1 is cleared, but not MAPT2 or MAPT4. This may also suggest cell death due to protein clearance failure at higher concentrations.

Reduction of LC3-II/LC3-I by Nystatin at 0.48μg/mL within CV4a could indicate normal autophagy function but there was a significant increase in only CV17q MAPT accumulation which might be due to abundance of protein in the sample or special
accumulation of tau as compared to CV4a. CV17q may be more sensitive to drug treatments, but in order to evaluate this outcome, the number of cells and/or total protein must be increased in the CV4a cell line.

Cell viability decreased with PuAD treatment at higher concentrations (1200nM; 420nM) in both cell lines. This is not consistent where viability of iPSC-derived neurons showed little change after 72hr treatment with PuAD in concentrations ranging from 0-100 uM (Inda et al. 2020). Concentrations of PuAD in the present study were significantly higher and could be the cause of increased cell death. Inhibition of HSP90 affects the ubiquitin proteasome system where a reduction in LC3-II/LC3-I may indicate autophagy activation. The increase in MAPT4, MAPT5, and total tau within CV4a and additionally, increase of all tau protein by 420nM and an increase of MAPT1, MAPT2, MAPT4, and total tau by 51.45nM in CV17q could be the result of normal autophagy function with no mechanism for degradation of these MAPT proteins (Inda et al. 2020).

Treatment with Vorinostat may not affect autophagy function but all tau measurements indicate increased tau accumulation at least in the CV17q cell line. CV4a follows the trend of increasing tau protein, however this could be due to Vorinostat having less of an effect on healthy control cells. Loaded Western blots with Vorinostat treated samples may be skewed due to an obstruction caused by the tau protein ladder with bleeding signal through adjacent wells. Data cannot be confirmed here and further studies are required.
Profile Analysis of Gene Expression Between 2D vs 3-D, in Control vs “Diseased” Cell Lines

Comparisons were made between CV4a and CV17q cell lines where identification of cell type (neurons, astrocytes, and oligodendrocytes) specific genes may yet elucidate gene expression profiles for these cell lines. Based on the bioinformatics technique proposed in Cahoy et al. (2008) the selected genes (Table S1) were expected to be expressed in neurons: (1)Neurod6, (2)Vip, (3)Gabra5, (4)Satb2, (5)Sla, (6)Odz2, (7)Crh, (8)Syt1, (9)Dlx1, (10)Pgm2l1 (11)TUBA1A; (12)SYN1; Astrocytes: (1)Tnc, (2)Pla2g7, (3)Emp1, (4)Tgif2, (5)Ptx3, (6)Agxt2l1, (7)Tcfcp2l1, (8)Dmp1, (9)Sult1a1, (10)Gjb6; Oligodendrocytes: (1)Mobp, (2)Cpm, (3)Adamts4, (4)Enpp6, (5)Mog, (6)Ugt8a; and constitutive housekeeping genes: (1)GAPDH, (2)Tbp, (3)RPL27, (4)EEF1A1.

Differences in gene expression that are described in the results (Figure 24) are seemingly contradictory to my hypothesis that 3-D neural spheres are comparable as an in vivo model however, non-adhered 3-D cultures have proven difficult to manage. Any disturbance to the cultures such as weekly media changes caused a loss of some cells or entire cultures. The fragility of the cultures requires a skillfulness acquired by proper training and experience (lab experience) by which one could efficiently reproduce the experiment with improved results.

A major component of this project was to specifically monitor levels of total, 3R, and 4R tau protein within cell cultures. Because tau is expressed in most human central nervous cells and primarily expressed in neurons (Schraen-Maschke et al. 2008) my goal
was to elucidate different isoform expression (3R and 4R tau) compared to total tau to
determine if cells generate higher expression of one isoform over the other. Results
established that only 3R tau was significantly increased in my CV17q 3-D model when
compared to CV4a (3-D) neural spheres. These results could be due to the amplification
region included in the CV17q cell line.

Integration of Cell Type Specific EGFP Induced Plasmid Cassettes and Fluorescent
Microscopy

Cell type specific fluorescent reporters at the AAVS1 safe harbor locus have been
developed and stably integrated in tauopathy-like and healthy control lines. The
construction of fluorescent reporter plasmid tools includes cell type specific promoters
and EGFP to distinguish individual cells within each cell population. PRNP, SYN1,
GFAP, CNP, and EEF1A1 plasmids have been constructed and gel electrophoresis has
confirmed the presence of the correct plasmid amplification sizes (Figure 5). The goal at
this stage was to identify the key cell populations that are negatively affected by
tauopathies (oligodendrocytes, astrocytes, neurons) and to compare the results of these
reporter studies to our gene expression profiles at various stages of differentiation. EGFP
labeled cells allowed for differences in gene expression to be visualized by fluorescence
microscopy and immunofluorescence. As cells differentiate, cell signaling and gene
expression determine cell fate. Although there are many cell type specific genes
expressed at any developmental stage, any gene may be activated at any point across
development. Observed EGFP expression may be due to cell type specific gene
expression, but if at any point a cell expresses a reporter gene within its relative condition, that promoter then influences the expression of EGFP. A neural progenitor cell may become a neuron, but in order to become a neuron, any number of genes in any combination could be regulated and/or expressed during the differentiation process until that cell’s fate is determined. Problems/pitfalls of this aim could include unpredicted expression of EGFP.
SUMMARY AND FUTURE DIRECTION

Deaths associated with tauopathies are still considered "incurable"; therefore, construction of novel preventive technology must be fully explored. A large-scale production of distinct cell types affected by a specific disease would be useful for disease modeling, drug discovery, and autologous cell replacement therapies where the possibility of prevention should be examined as an alternative to the direct assault on proteins through medicating.

Mapping distinct cell signaling pathways and tracking their reactions over time during specific stages of a life cycle via gene expression and protein markers are a rising trend in the field of biology and bioinformatics (Zhou et al. 2016). Gene function/expression in individual cells and/or whole cell lineages may be regulated with the potential to survey one or multiple cell signaling pathways at a time. The configuration and development of experimental cell lines based on biochemical signaling in the presence of certain transcription factors would distinguish gene function within individual cell types. Rather than relying on the continued use of medications that have demonstrated minor efficacy for treating tauopathies, future direction will target upstream signaling and post-translational modifications for novel treatment and prevention strategies. Enhanced cell culture techniques may have the potential of improving the data from the neural models studied in this project. It remains unconvincing to me that the results obtained from the 3-D neural sphere cultures are accurate depictions of their gene expression. Due to the obstacles presented when performing cell culture techniques and
media changes, re-adherence of 3-D neural spheres might reduce the probability of damage to the scaffolding structures and loss of sample, thus increasing the likelihood of improved neural modeling (Figure. 33).

Figure 33. Bright field micrographs of 2D monolayer (left) and 3-D neural spheres (right). Cells displayed here were cultured from CV17q iPSC derived neural progenitor cells and further differentiated. (Left) Bright field microscopy shows the monolayer cell cultures, traditionally used in neural developmental research. (Right) Bright field microscopy showing different growth stages of differentiated neural spheres. Axons and dendrites can be more easily distinguished in the 3-D cultures as well as obvious connections that are being made between cells.

Rather than relying on the continued use of medications that have demonstrated minor efficacy for treating tauopathies, future direction will target upstream signaling and post-translational modifications for novel treatment and prevention strategies. A stably integrated cell type specific fluorescent reporter at the AAVS1 safe harbor locus in tauopathy-like and healthy control lines has been developed. In future projects, these tools can be coupled with a CRISPR system to systematically dissect cell type specific phenotypes in high resolution using modern genetic tools. It would be helpful to use CRISPRs in order to: (1) insert these tools at a known locus in the human genome without interference with other genes; (2) control for copy number and expression level; and (3) avoid experimental heterogeneity associated with random viral integration or poor transient transfection rates.
Whether native and/or misfolded tau is cleared and recycled by specific degradative pathways must still be investigated. Autophagosome formation genes, as well as ubiquitin cargo recruitment genes can be targeted by sgRNA sequences to initiate gene interference and/or activation by the plasmids constructed in this experiment with the iCRISPRi/dCas9 system (Figure. 2; Figure. 5). The regulation of autophagic mechanisms by enhanced drug screening and/or gene expression could identify and confirm autophagosome and/or ubiquitin binding inhibition. iCRISPRi constructs could be integrated into the AAVS1 safe harbor locus of these isogenic human iPSC lines and knockdown by cell specific promotor control, targeting autophagosome formation genes and ubiquitylated cargo recruitment genes could confirm mechanisms in drug induced intervention. This will also provide a different type of (drug) treatment as a possible prevention rather than the present search for a cure. Neural development and survival are dependent on constitutive autophagy, gene regulation and cell signaling; and the dysregulation of these functions leads to neurodegeneration. Understanding these pathways will allow for safe and more effective therapies for neurodegenerative patients.
REFERENCES/LITERATURE CITED


