Characterization of SH-SY5Y cells after differentiation using retinoic acid and brain-derived neurotrophic factor

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by

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Abstract:

Previous studies (Encinas et al., 2000) have designed a protocol to derive neuron-like cells from the human neuroblastoma cell line SH-SY5Y using sequential exposure to retinoic acid (RA) and brain-derived neurotrophic factor (BDNF). These studies focused primarily on nonspecific neuronal markers to indicate the presence of fully differentiated neuron-like cells, without addressing the question of neurotransmitter phenotype or the substrate upon which cells were differentiated. The purpose of this experiment was to optimize the induction of a dopaminergic phenotype in SH-SY5Y cells by varying the differentiation substrate. For differentiation experiments, cells were treated sequentially with retinoic acid and BDNF after being plated on either bare plastic or collagen coated plates. Cells were harvested and pictures were taken at 0 hours or 72 hours after the start of differentiation. Morphological analyses of the percent of differentiated cells and average neurite lengths were used to determine general levels of differentiation. Biochemical phenotyping through qPCR of mRNA transcripts determined the level of dopamine specific expression as well as other neurotransmitter phenotypes. Primers were directed against human tyrosine hydroxylase (TH), dopamine active transporter (DAT), choline acetyltransferase (ChAT), serotonin transporter (SerT), norepinephrine transporter (NET); and control genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal subunit protein (RPL19). Results indicate that treatment with RA and BDNF results in high levels of morphological differentiation regardless of differentiation substrate. RA and BDNF treated cells have slightly longer average neurite lengths on collagen-coated plates; however, this difference was not statistically significant. DAT was expressed at a basal level in all cells; however, in all experimental cases DAT expression decreased after 72 hours. DAT expression decreased the most in cells differentiated on collagen and the least in cells differentiated on
plastic. Also, GAPDH expression varied less than RPL19 expression between differentiated and undifferentiated cells.

To summarize: during differentiation using RA and BDNF, differentiation on collagen-coated plates rather than bare plastic does not affect the number of cells with differentiated neuronal morphology, or the expression of the dopamine specific marker, DAT. Collagen coating may increase the average neurite length. Future directions of this study will further examine the neurotransmitter phenotype of differentiated SH-SY5Y cells. Examination of other phenotypic effects will be done using morphogens such as trolox, as well as other ECM molecules such as laminin or poly-d-lysine.
Introduction:

Dopaminergic neurons are central in the etiology of neurological disorders including Parkinson’s disease and drug addiction (Kovalevich and Langford, 2013). Parkinson’s disease is characterized by a neurodegenerative loss of dopaminergic or dopamine utilizing neurons in the brain (Nagatsu et al. 2019). At the system level, loss of dopamine neurons leads to a dysfunction of basal ganglia motor circuits, resulting in the symptomology characteristic of Parkinson’s. One major hypothesis among Parkinson’s researchers proposes that dysfunction in dopamine handling and/or mitochondrial integrity within dopamine neurons leads to the formation of reactive oxygen species, which results in protein aggregation and cell death (Puspita et al., 2017).

Early-onset forms of Parkinson’s disease have been studied and genes related to dopamine neurotransmission and oxidative stress (e.g. alpha-synuclein and LRRK2) have been identified as causes, but few breakthroughs on the pathology of sporadic onset Parkinson’s disease have been made (Wallings et al. 2015, Butler et al. 2017, Polymeropoulos et al. 2019).

Research has shown that decreased expression of an enzyme known as tyrosine hydroxylase (TH) has been associated with the physiopathology of Parkinson’s disease that results from the degradation of neurons in substantia nigra region of the brain that help regulating motor function (Nagatsu et al. 2019). It is important to note that these neurons of the substantia nigra are characterized by their use of dopamine as a neurotransmitter, making them catecholaminergic. A key biomarker in all catecholaminergic neurons, or neurons that utilize dopamine and/or norepinephrine, is the presence of the TH enzyme. TH is involved in the reaction that converts L-tyrosine to L-3,4-dihydroxyphenylalanine (L-Dopa). This reaction is the first step in catecholamine biosynthesis and it also the rate limiting step of the whole reaction. Small changes to the level of TH in catecholaminergic neurons can have drastic effects on the
levels of dopamine and norepinephrine and ultimately the function of these neurons (Nagatsu et al. 2019). Further down the pathway for catecholamine biosynthesis is an enzyme known as DOPA decarboxylase. The DOPA decarboxylase enzyme is responsible for the catalysis of the conversion of DOPA into dopamine. Dopamine is then converted into norepinephrine by the action of dopamine-β hydroxylase. Lastly, Epinephrine is formed from norepinephrine with the enzyme phenyl ethanolamine N-methyl-transferase. The phenotype of each cell is determined by the transcriptional regulation of each of these enzymes. A dopaminergic neuron will be characterized by the expression of DOPA decarboxylase but not the further downstream proteins involved in the formation of norepinephrine and epinephrine (Purves et al., 2018). Following packaging of catecholaminergic neurotransmitters into vesicles via the common vesicular monoamine transporter, VMAT2 (Erickson et al., 1992, Surratt et al., 1993). After neurotransmitter release, reuptake of dopamine is mediated through the plasma membrane dopamine transporter (DAT) while reuptake of norepinephrine is mediated through the norepinephrine transporter (NET). Dopamine is a substrate of both transporters; however, the transporters differ substantially in their expression patterns, structures, and binding partners \textit{in vivo} (Sager and Torres, 2011). With this understanding we can adequately characterize a cell’s phenotype based off the expressing of metabolic proteins. Although transcriptional profiling is useful and is the main discussion in this paper, it is possible to measure neurotransmitter levels and metabolites of neurotransmitter synthesis directly using methods such as high-performance liquid chromatography (HPLC).

In order to further examine a putative link between Parkinsonism and cellular mechanisms of catecholamine biosynthesis, packaging, release, and reuptake, it is common practice to use cell lines as models. Cell lines allow researchers to study dopaminergic neurons
without having to acquire costly primary neuron samples. Cell lines also can facilitate the use of human cells in research, without having to harvest human brain tissue. The cell line SH-SY5Y, isolated from a human neuroblastoma, is commonly used in neurobiology research as a dopaminergic model (Kovalevich and Langford, 2013). The SH-SY5Y cell line is commonly used as a dopaminergic model for Parkinson’s disease in neurotoxicity assays such as 3,4-Catechol-PV in MDPV-induced neurotoxicity and MPP+ uptake assays (Coccini et al. 2019), (Korecka et al. 2013). Many of the cytotoxic chemicals involved in these assays are catecholamine derivatives and enter the cell via monoamine transporters. The findings from these assays indicate that catecholamine metabolism and uptake is significantly affected by cytotoxic chemicals such as 3,4-Catechol-PV or MPP+. However, applying the results from these experiments to the investigation of Parkinson’s disease etiology or pharmacotherapy relies on the assumption that SH-SY5Y cells are a good model of dopaminergic neuronal function. SH-SY5Y cells originate from the neural crest tissue, which drives their differentiation toward a catecholaminergic phenotype but can also give rise to many other cell types (Encinas et al. 2000). Exposing these cells in culture to various morphogens and growth factors causes the cells to differentiate into neuron-like cells both morphologically and transcriptionally.

The protocol we are interested in was first reported by Encinas et al. 2000, and it was reported to produce large populations of homogeneous human neuron-like cells free of other neural crest-derived cells. This protocol uses co-administration of retinoic acid (RA) with the neurotrophin brain-derived neurotrophic factor (BDNF) to induce differentiation of SH-SY5Y cells. In this study researchers looked at the sequential treatment of SH-SY5Y cells with RA and BDNF. BDNF had a positive effect on the formation of fully differentiated neuron-like cells, both on expression of general neuronal markers and on neuronal morphology. The same study
showed that RA caused SH-SY5Y cells to come out of the cell cycle and stop dividing, while also expressing neuronal markers such as neurofilament light peptide (NF-L), neurofilament medium peptide (NF-M), and neuron specific enolase (NSE). The current study aims to manipulate the presence of RA and BDNF in cultures, to determine what neurotransmitter phenotypes the differentiated cells are adopting, and to select for a protocol that yields the highest expression of dopamine-specific markers.

A variety of morphogens have been reported to induce differentiation in SH-SY5Y cells, including retinoic acid, trolox, phorbol esters, estradiols, and cholesterol compounds (da Fronta et al. 2011; Pahlman et al., 1984, Teppola et al. 2016). Retinoic acid (RA) is a commonly-used morphogen in almost every SH-SY5Y differentiation protocol. RA is a vitamin A derivative and controls the function of genes involved with stopping cell growth and division, inducing cell differentiation, and regulating metabolic genes through intracellular signaling involving retinoic acid receptors (Rochette-Egly 2015). Inactive retinoic acid receptors are typically monomers that dimerize when bound to RA. This dimer acts as a transcription factor that localizes to the nucleus to promote the expression of the retinoic acid response element (RARE). The RARE regulates the transcription of genes that limit cell growth and division, induce transcriptional changes that lead to differentiation, and regulate metabolism. RA-treated cells are commonly used as a model for dopaminergic neurons; however, they have been reported to adopt a dopaminergic, noradrenergic, or even cholinergic phenotype (Pahlman et al., 1984; Xie et al., 2010; Korecka et al., 2013). Previous research demonstrates that using RA favors the induction of a catecholaminergic phenotype (Xicoy et al., 2017; Encinas et al., 2000). Some researchers have suggested simultaneous treatment with RA and other growth factors produces a stronger dopaminergic phenotype after differentiation (Kovalevich and Langford, 2013). Experiments
have been conducted in line with the current study that aim to phenotype the SH-SY5Y cell line by casting a wide net on possible neurotransmitter phenotypes via microarrays for gene transcription profiling (Korecka et al. 2013). However, the validity of microarray analysis of gene expression has come into question within recent years (Nadon and Shoemaker, 2002). Microarrays are prone to statistical invalidity on account of their relatively low sample sizes and their high levels of variables. The current study utilizes a more validated process of qPCR to determine gene expression. The lack of clarity regarding the neurotransmitter phenotype of RA-treated SH-SY5Y cells makes it necessary to determine the expression patterns of markers for neurotransmitter phenotype in RA-differentiated cells.

In general, it is believed that TH in expression in SH-SY5Y cells is good indication that they are catecholaminergic cells because TH provides these cells with the mechanisms to produce both dopamine and norepinephrine (Xicoy et al. 2017). According to Encinas et al. 2000, and many more recent papers that utilize the same commonly-used RA and BDNF based differentiation protocol, SH-SY5Y cells do become catecholaminergic cells after differentiation based of TH expression (Dwane et al. 2013; Kovalevich and Langford 2013; Xie et al. 2010). However, there is controversy over the use of these malignant cells as a dopaminergic model because of the fact that they may be characterized by more than one neurotransmitter phenotype. The lack of clarity regarding the neurotransmitter phenotype of RA-treated SH-SY5Y cells makes it necessary to determine the expression patterns of markers for neurotransmitter phenotype in RA-differentiated cells.

Not all previous research conducted in the SH-SY5Y cell line has specifically considered induction of a dopaminergic phenotype. Characterizations have been made using general neuronal markers and morphological analysis only, without examining the level of dopaminergic
phenotype in differentiated cells (Encinas et al. 2000). Tyrosine hydroxylase (TH) can be used as a general marker for catecholaminergic neurons because TH is the rate limiting enzyme in catecholamine biosynthesis (Nagatsu et al. 2019). However, analysis of separate marker genes is necessary to better determine what neurotransmitter phenotypes are expressed in differentiated cells. In order to ensure that model systems are appropriately used, researchers must understand both the morphological and the biochemical differentiation of neuron-like cells. We know that in vivo, these cells come from a neuroendocrine tumor in the adrenal gland that derives directly from the neural crest cell population in a developing embryo (Tsokos M et al. 1987). Non-malignant cells derived from this lineage typically go on to produce the trunk neural crest and develop into the sympathetic preganglionic cell population. These cells utilize norepinephrine and are expected to produce the plasma membrane norepinephrine transporter (NET) naturally (Laskey and Polosa 1988). However, SH-SY5Y cells arise from a malignant tumor, and can therefore be expected to display significant alterations in their transcriptional regulation. Furthermore, in a controlled lab setting the presence of morphogens and growth factors is limited and controlled. Therefore, a more rigorous analysis of differentiated SH-SY5Y cell phenotype is required. We aim to characterize SH-SY5Y cells based on an array of possible neurotransmitter phenotypes.

Another important factor in the differentiation of cells is the presence of extracellular matrix (ECM) molecules. Both the morphology and the expression of neuron-specific genes are affected by the physical substrate upon which cells are differentiated (Li et al., 2007). ECM molecules not only provide structure for developing neuronal cells, but also interact with cells though various signaling pathways (Li et al., 2007). Specifically, integrins regulate the growth of neurites by interacting extracellularly with ECM molecules. Integrins, when associated with
other growth factors, allow the SH-SY5Y cell line to fully differentiate both transcriptionally and morphologically (Li et al., 2007). Previous research indicates that the longest and most numerous neurite growth is observed in SH-SY5Y cells grown on laminin and collagen coated plates (Dwane et al., 2013). Previous studies also indicate that collagen affects the morphology of differentiating neural crest cells by increasing the length and number of axonal and dendritic projections (Lallier et al., 1992). Additionally, collagen provides a more affordable solution for ECM coating plates than other commonly used materials like fibronectin, poly-d-lysine, or laminin.

In the research reported here, we anticipated that treatment of SH-SY5Y cells with RA and BDNF would give rise to neuron-like cells with a dopaminergic phenotype, expressing both TH and the plasma membrane dopamine active transporter (DAT). We also hypothesized that collagen-coated plates would yield the highest upregulation of our marker genes because collagen may act as a scaffold for neurite outgrowth and enhance the neuronal morphology of differentiating sympathetic neurons, which are derived from the same precursor population as SH-SY5Y cells (Lallier et al. 1992). We evaluated morphological differentiation by quantifying the number and length of neurites and categorizing and quantifying overall cell morphology. Morphological analyses were done using data from two separate differentiation experiments performed on separate days. We also determined the neurotransmitter phenotype of differentiated and non-differentiated cells by used real-time PCR (qPCR) to gather semi-quantitative values for gene expression of various marker neurotransmitter markers. qPCR utilizes a dye in the qPCR reaction mix that binds in between base pairs of newly synthesized DNA and becomes fluorescent when bound. We measure critical threshold values (Ct) which are the number of cycles it takes for the fluorescence in a qPCR well to reach the threshold of detection of the
qPCR machine. Samples with higher input, or higher levels of transcription for a given gene, reach the florescent detection threshold sooner and will have smaller C\textsubscript{t} values. Inversely, samples with less input, or lower levels of gene expression, will have higher C\textsubscript{t} values as it will take more cycles to reach the threshold of fluorescence detection. This process for semiquantitative gene has been validated, and our experiment follows the minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Taylor et al. 2010). All of our qPCR reactions were run in triplicate on a total of 16 separate culture plates from two separate differentiation experiments. In our qPCR analysis we measured expression of the catecholaminergic marker TH and the dopaminergic-specific marker DAT. We also examined marker gene expression for other neurotransmitter phenotypes common in sympathetic neurons, including NET and the cholinergic marker choline acetyltransferase (ChAT). Finally, we examined expression of the serotonin transporter (SERT), a closely-related gene to DAT and to NET that is not expected to be expressed at high levels in catecholaminergic neurons. By selecting and measuring multiple neurotransmitter phenotypes we will be able to better characterize SH-SY5Y cells after differentiation using the RA/BDNF protocols.

**Methods:**

*Cell Culture and Maintenance*

The human SH-SY5Y cell line was purchased from ATCC (CRL-2266). Cells were cultured in untreated T-25 flasks (Corning) and maintained at 37°C, 5% CO\textsubscript{2}, and saturated humidity in proliferation medium (Dulbecco’s Modified Eagle’s Medium/F12, 10% fetal bovine serum, 1% penicillin-streptomycin solution) (Sigma: D0547, F2442, P4333) at pH 7.4. Cells were initially plated at about 20-30% confluency. We permitted cells to proliferate until about
90% confluency, then passaged them using 0.25% trypsin (Sigma T4049) to detach cells from flasks. Passaging was used as a way to control cell density by plating at lower densities of cells. Cells used for differentiation experiments underwent between 8 and 10 rounds of proliferation and passaging.

**Differentiation in Culture**

Cells were passaged as described above and plated at density of 400 cells/cm² on 10 cm culture dishes (Corning 430167), either collagen-coated (Sigma C8919) or untreated (i.e., plated on bare plastic). Plates were collagen coated using a suspension of type I collagen in sterile water. The final concentration of collagen on plates after drying was 8ug/cm². A portion of passaged cells was harvested immediately after passaging with trypsin, and used as a negative control (Time 0). Once plated, the cells receiving differentiation treatment were covered in 10 mL of differentiation medium 1 (DMEM/F12 (Sigma D8900), 1% FBS (Sigma F2442), 1% pen-strep (Sigma P4333), 10 µM all-trans retinoic acid (Sigma R2625)) for 24 hours. The negative control cells were covered in differentiation medium 1 without retinoic acid (RA). After 24 hours, differentiation medium 1 was replaced with differentiation medium 2 (DMEM/F12, 1% pen-strep, 50ng/ml BDNF (Sigma B3795)) in experimental groups. The negative control groups received differentiation medium 2 without BDNF. 48 hours after the medium change the cells were harvested, pelleted by centrifugation for 5 minutes at 1500xg, and stored at -80°C. Pictures were taken of cells at 0 hour, 24 hours, and 72 hours (**Figure 1**) with a Motic WI-FI camera mounted on an American Optic series 1820 Bio-star inverted light microscope using a custom-fabricated adaptor under a total magnification of 100x.
Harvesting SH-SY5Y cells

Plates were removed from the incubator and rapidly cooled on ice. The culture medium was removed via aspiration and discarded, and 3 quick and consecutive washes with 1 milliliter of ice-cold phosphate buffered saline (PBS, pH 7.4) were performed. Another 1 milliliter of PBS was added to the plates and the cells were scraped using Greiner cell scrapers (Sigma C5981). The cell suspension was centrifuged, and a pellet was collected and stored at -80°C.

RNA isolation and cDNA synthesis

Cell pellets were thawed, and RNA isolation was performed using Qiagen RNeasy kits (Cat No. 74104) according to the manufacturer’s protocol for animal cells. RNase free equipment and technique were used to prevent RNA transcript degradation. RNA concentration and integrity were assessed using agarose gel electrophoresis and ethidium bromide staining. cDNA synthesis was performed using the Applied Biosystems™ High-Capacity cDNA Reverse Transcription Kit (ThermoFisher 4368814) and protocol. RNase inhibitor was not used in the reaction mix.

qPCR Primer Design

Primers were designed using NCBI Primer BLAST. All primers used in qPCR analysis were designed to produce a product between 70-150 bp in length, and to cross at least one exon-exon boundary as a safeguard against any potential genomic DNA contamination of the cDNA libraries. Primers were designed to nonspecifically amplify different transcriptional variants of those marker genes that are alternatively spliced and/or have alternate transcriptional start sites.
Our primer design strategy used the default Primer BLAST settings for primer melting temperature ($T_m$) and for assessment of primer specificity within the human transcriptome. Each primer was tested using RT-PCR and agarose gel electrophoresis with ethidium bromide staining before qPCR was performed in order to verify it generated amplicons of the expected size. Table 1 includes all qPCR primers used in the study, including primers designed by the author.

Real-time PCR

Primers were validated based on melt curve data. Primers that showed consistent melt curves between samples and had only one definite peak were considered valid. Primer sets that failed to provide consistent curves or had more than one peak in each curve were discontinued in further experiments. PCR reactions were run in triplicates in order to remove any outliers that could have resulted from variations in pipetting. Any $C_t$ values that were more than 2 cycles away from the other two in a sample were discarded and assumed to be a result of unequal pipetting. Outliers that were more than 3 standard deviations from the mean were also removed. There were only 4 $C_t$ values classified as outliers that were removed.

Real-time PCR was performed using iTaq™ Universal SYBR® Green Supermix (Bio Rad 1725121) and a Bio Rad CFX96 Touch™ Real-Time PCR Detection System with cDNA template. Each well had a total reaction volume of 10 µL. Reactions for each primer were performed in triplicate according to the Bio-Rad protocol. The primers are detailed in Table 1.

Data Analysis

Morphology was assessed in two separate analyses of cell photographs. Images from two separate experiments done on different days were used. Categorization was not blinded to treatment. In the first method, cells were categorized as either differentiated (neuronal
morphology) or non-differentiated (fibroblast or spindle morphology). Fibroblast morphology was characterized by squamous shape and lack of neurite outgrowth. Spindle-shaped cells had collapsed and rounded cell bodies with projections at opposing poles. Neuronal morphology was characterized by a distinct round cell body and neurite outgrowth. We used the chi-squared test to determine the statistical significance of any differences in the frequency of observed morphologies between treatment groups.

We also examined neurite outgrowth by measuring the average length of a single cell’s neurites. NIH Image J software with the “simple neurite tracer” plugin was used to measure the total length of neurites in a field of view. Experimenters were blinded to treatment and surface conditions during the neurite analysis. We validated our ability to measure using this protocol by tracing circles and boxes of known sizes and comparing them to our results. We required scorers to be within 5% of the actual values for 3 separate trials to validate their ability to score, Standard for measurement can be found in Figure 1. Photos were converted from RGB images to 8-bit luminance images in Image J software before using the plug-in. Only neurites clearly connected to a cell body in the frame of the image were traced. The total length of all traced neurites was divided over the total number of cells in the frame to get an average neurite length per cell. Differences in neurite length between groups were assessed using 2-way analysis of variance (ANOVA) on SPSS software, with differentiation condition and growth substrate as the main factors. Post-hoc testing, where warranted, was done using the Bonferroni method.

Gene expression was analyzed by calculating the fold change in mRNA abundance using the double ∆Ct method (Livak and Schmittgen, 2001). Expression of probe genes is quantified using the formula ∆Ct in which expression is normalized to reference genes (RPL19 or GAPDH):

$$\Delta C_t = (\Delta C_{\text{probe}} - \Delta C_{\text{ref}})$$
Changes in gene expression between an experimental and a control group were determined using the formula $\Delta \Delta C_t$:

$$\Delta \Delta C_t = (\Delta C_{exp} - \Delta C_{control}).$$

Fold change was expressed as $2^{-\Delta \Delta C_t}$.

Hypothesis testing for between-group differences was analyzed using one-way Analysis of Variance with Bonferroni post-hoc testing to determine significant differences in gene expression between individual sample groups. This process was done for each gene of interest.

**Results:**

**Morphological Phenotype**

**Cell categorization**

For all morphological data we used pictures from 0 and 72 hours post differentiation from two separate experiments. Sample size information is given in Table 2. We demonstrated that treatment with RA and BDNF resulted in an increased percentage of differentiated cells on all surfaces (Figure 2). Control groups did not yield any fully differentiated cells on plastic or collagen. Spindle morphology was transiently observed in many cells 24 hours after RA treatment, but this morphology gave way to fully-differentiated morphology by the 72-hour time point and was therefore not quantified. Of the total cells that were differentiated, 48.5% displayed mature neuronal morphology on plastic dishes after 72 hours. Of the total cells differentiated, 51.5% displayed neuronal morphology on collagen after 72 hours. 0% of the control cells show neuronal morphology at 0 hour or 72 hour timepoints. There is not a significant difference in the percentage of differentiated cells on collagen versus plastic ($p = 0.102$).
The analysis of cell categorization is complicated by the fact that cell populations behave differently under each of the experimental conditions. Different treatment groups may have produced different numbers of cells. Some may have caused cells to undergo apoptosis, to exit from the cell cycle at various times, or to alter the rate of mitosis. In order to obtain the expected proportion of differentiated cells in each treatment group (assuming the null hypothesis to be true) we used the formula:

$$\text{expected} = n \times (P_D \times P_o)$$

where $n =$ total number of cells counted in the experiment, $P_D =$ the proportion of differentiated cells counted in the experiment, and $P_o =$ the proportion of cells from each treatment group counted in the experiment. The actual proportion of differentiated cells sorted by each group was determined by counting the number of differentiated cells in each treatment group and dividing by the total number of cells counted in the experiment.

The percentage of differentiated cells that we observed in each group, vs. the percentages expected if the null hypothesis were correct, is shown in Figure 2. Chi-square analysis determined a significant difference ($p < 0.001$) between expected and actual values. As shown in Figure 3, 87.63% of the cells differentiated on collagen adopted a neuronal morphology and 90.24% of the cells differentiated on plastic plated adopted a neuronal morphology after 72 hours. Bonferroni post-hoc testing of the percentages of RA/BDNF treated cells on collagen coated plates and non-collagen coated plates demonstrated that this difference is not statistically significant ($p = 0.774$). There is no difference in the proportion of differentiated cells between collagen coated plates and bare plastic plates.
**Neurite Outgrowth**

The SH-SY5Y cells treated with RA and BDNF displayed positive outgrowth of neurites compared to untreated cells (Figure 4). We found that average neurite length was highest in RA/BDNF treated cells on collagen at 59.22 ± 4.78 µm. The average neurite length of cells differentiated on plastic was 57.43 ± 3.32 µm. Negative control cells, whether grown on plastic or on collagen, had no discernable neurite outgrowth outward on the plate. A two-way ANOVA determined that across the entire data set, differentiation condition significantly affected neurite outgrowth ($p < 0.001$). Substrate was not a significant factor in neurite length ($p = 0.833$). There were no statistically significant interaction effects between differentiation and substrate ($p = 0.833$). Bonferroni post-hoc testing showed no significant differences in average neurite length in the (RA/BDNF + collagen) group versus the (RA/BDNF without collagen) group ($p = 0.388$), between the bare plastic control group versus the (RA/BDNF without collagen) group, ($p < 0.001$) and the bare plastic control versus the (RA/BDNF + collagen) group ($p < 0.001$).

**Biochemical Phenotype**

**qPCR**

**Primers and Melt Curve analysis**

Melt curve analysis was performed on every primer set used in the experiment. Representative melt curves for each primer are shown in Figure 5 out of 48 total melt curves for each primer set. It is important to look at the definition of the peak in the melt curves when determining if primers are valid. Multiple peaks in a melt curve indicate the amplification of more than one transcript, and a non-specific primer (Ririe et al. 1997). Subthreshold melt curves are also an indicator of inefficient primers that have low binding affinity to their target (Ririe et
Curves for GAPDH, DAT, ChAT, SERT, and NET were very consistent showing a single strong peak, indicating that they are efficiently binding to cDNA and amplifying an amplicon of uniform length. Most of the curves for RPL19 also showed the same single peak, however there was more variation between samples. Melt curves for TH generally presented with sub- or near-threshold main peak values, and multiple peaks that were highly inconsistent. After the first qPCR plate, the use of TH primers was discontinued pending identification of a valid TH primer for use with the lab’s qPCR protocol.

**Normalization genes (GAPDH and RPL19) (Figure 6)**

GAPDH $C_t$ (# of cycles) values varied less between groups than RPL19 $C_t$ values. The line of best fit shows the variation in expression of RPL19 and GAPDH separately. Generally, a line with a slope closer to zero indicates a more reliable normalization gene. Normalization genes are characterized by their equal presence in both differentiated and undifferentiated cells. Ideally a normalization gene will be expressed at the same level in both cases; thus, analysis of the gene expression before and after differentiation should yield a slope close to zero. There was no notable difference between GAPDH and RPL19 expression other than the difference in variance (n = 48 samples pooled across treatment conditions and experiments). Because there was slightly less variance in GAPDH expression, and in light of the results of our melt curve analysis, we chose to normalize expression of all genes to GAPDH. The normalized $C_t$ values for each of our genes is defined as:

$$C_t(GAPDH) - C_t(exp).$$

This value, which is identical to $\Delta C_t$ in the $\Delta \Delta C_t$ formula described in the *Methods*, was used in the one-way ANOVA to determine differences in mean $C_t$ value between treatment groups for
each gene. The ANOVA was selected to compare the distributions of the quantitative C\textsubscript{t} values across the qualitative variable of treatment.

**Tyrosine Hydroxylase (TH) expression (Figure 7)**

TH expression was not analyzed via qPCR because melt curve analysis determined that the primers for TH were invalid (Figure 5). Preliminary data from an earlier project in our lab confirms baseline expression of TH in SH-SY5Y cells via RT-PCR with detection of product by agarose gel electrophoresis (Figure 7). These results also suggested that TH expression increased following differentiation, based on visual inspection of band intensity; however, we were unable to perform hypothesis testing using qPCR to validate these results because our TH primer was itself invalid. Only one run of 12 wells was run before discontinuing the use of the TH primer.

**Dopamine active transporter (DAT) expression (Figure 8)**

Results indicate that DAT is expressed at basal level in undifferentiated cells with a 10.50 difference in C\textsubscript{t} values between DAT and GAPDH expression. Fold changes were calculated based on the expression of genes in the 0 hour control condition, which therefore is represented as having a fold change of 1. In all cases, DAT expression decreased over the 72 hour timeframe. The largest decrease in DAT expression was seen in the cells differentiated on collagen-coated plates, in which a fold change of 0.32 was recorded. The untreated cells at 72 hours experienced a 0.47 fold change in DAT expression. The smallest decrease in DAT expression was measured in the cells differentiated on plastic plates. The fold change in DAT expression in these cells was 0.69. However, results from a one-way ANOVA indicate that there is no statistically significant
difference in the mean of normalized $C_t$ values between treatment groups (n = 14 samples in 3-4 separate culture wells per group, $p = 0.545$).

**Choline Acetyltransferase (ChaT) Expression (Figure 9)**

0-hour control cells showed basal expression of ChAT with a 10.42 difference in $C_t$ values between ChAT and GAPDH at time 0. By 72 hours, control cells and differentiated cells on collagen appeared to have downregulated ChAT expression (0.82-fold and 0.48-fold changes, respectively). Differentiated cells on plastic plates showed a slight upregulation of ChAT compared to time 0 control cells (1.15-fold). Results from a one-way ANOVA indicate that there is no statistically significant difference in the mean of normalized $C_t$ values between treatment groups (n = 14 samples in 3-4 separate culture wells per group, $p = 0.847$).

**Serotonin Transporter (SERT) Expression (Figure 10)**

Results for SERT expression indicate that SH-SY5Y cells express basal levels of SERT pre-differentiation. There is a 9.78 difference in $C_t$ values between SERT and GAPDH at time 0. SERT was downregulated in all experimental conditions after 72 hours. Untreated cells at the 72 hour timepoint demonstrated a 0.27-fold change in SERT expression. Cells differentiated on collagen showed a 0.19-fold change in SERT expression. Differentiated cells on plastic showed a 0.63-fold change in SERT expression. Results from a one-way ANOVA indicate that there is no statistically significant difference in the mean of normalized $C_t$ values between treatment groups (n = 14 samples in 3-4 separate culture wells per group, $p = 0.105$); however, this $p$-value is approaching statistical significance, implying that there may be a true downregulation of SERT post-differentiation, which our experiment may have lacked sufficient statistical power to detect.
**Norepinephrine Transporter (NET) Expression (Figure 11)**

qPCR analysis shows basal expression of NET in SH-SY5Y cells. There is a 8.61 difference in $C_t$ values between NET and GAPDH at time 0. Results from qPCR analysis of NET expression appeared to show upregulation of NET across all treatment conditions after 72 hours. Untreated cells upregulated NET expression by 6.18-fold at the 72-hour time point, the largest increase in NET expression level of all the groups. Differentiated cells on collagen increased NET expression by 4.66-fold, and differentiated cells on plastic increased NET expression by 5.75-fold. Despite the magnitude of this difference, there was significant variability in NET expression within groups, and results from a one-way ANOVA indicate that there is no statistically significant difference in the mean of normalized $C_t$ values between treatment groups ($n = 14$ samples in 3-4 separate culture wells per group, $p= 0.110$).
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<tr>
<td>RPL19</td>
<td>60S ribosomal protein L19</td>
<td>TCGCCTCTA GTGTCCTCCG</td>
<td>GCGGGCC AAGGTGTT TTTC</td>
<td>6143</td>
<td>99</td>
<td>NM_000981 NM_0013302001</td>
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<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
<td>CCTGACCT GCCGTCTA GAAA</td>
<td>GAGTGGGT GTCGCTGT TGAA</td>
<td>2597</td>
<td>114</td>
<td>NM_002046.7 NM_001289745.2 NM_001357943.1 NM_001289746.1 NM_001256799.2</td>
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<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
<td>GGACCTCC ACACTGAG CCAT</td>
<td>TTACCATG ATGGCCTC TGCC</td>
<td>7054</td>
<td>112</td>
<td>NM_199292.2 NM_199293.2 NM_000360.3</td>
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<tr>
<td>DAT</td>
<td>Dopamine Active Transporter</td>
<td>CACCATGC CATACGTG GTC</td>
<td>CCGTGAAG TCAACCG CTCA</td>
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<td>103</td>
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<tr>
<td>ChAT</td>
<td>Choline Acetyl Transferase</td>
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<td>CCCGAATT TCCAGAGG TCGG</td>
<td>1103</td>
<td>95</td>
<td>NM_020985.3 NM_020986.3</td>
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<td>SERT</td>
<td>Serotonin Transporter</td>
<td>GCGTGCAA CCCGACGA TAG</td>
<td>ACACTGAT GTCCATCT GCCA</td>
<td>6532</td>
<td>92</td>
<td>NM_001045.6</td>
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<tr>
<td>NET</td>
<td>Norepinephrine transporter</td>
<td>CTTCCCCCTA CCTCTGCTA CAAGAA</td>
<td>CCCGCATA AGGAACAG</td>
<td>6530</td>
<td>112</td>
<td>NM_001172504.1 NM_001172501.1 NM_001043.3</td>
</tr>
</tbody>
</table>

*Table 1* shows each primer set used in the experiment. Including Forward primer sequence, reverse primer sequence NCBI gene ID and the expected amplicon size. Primer Harvard bank was the source for SERT primers (Primer Bank ID: 225007595c2). NCBI primer finder was used to custom design RPL19, GAPDH, TH, DAT, ChAT, and NET primers for this study.
**Figure 1.** Representative images of SH-SY5Y cells 72 hours after the start of experiment using phase optics, at 100x magnification using a Motic Wifi camera. 

- **A:** Control 0 Hr
- **B:** Control 24 Hr
- **C:** Control 72 Hr
- **D:** Control +collagen 72 Hr
- **E:** RA/BDNF 72Hr
- **F:** RA/BDNF +collagen 72 Hr

Differentiated cells (C,D) adopt neuronal morphology characterized by formation of neurites. 

**G:** Standard for neurite measurements and validation (100 um)
<table>
<thead>
<tr>
<th>Group</th>
<th># of Pictures</th>
<th># of cells</th>
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</thead>
<tbody>
<tr>
<td>Control 0 Hour</td>
<td>7</td>
<td>312</td>
</tr>
<tr>
<td>Control 72 Hour</td>
<td>12</td>
<td>183</td>
</tr>
<tr>
<td>Control +Collagen 72 Hour</td>
<td>6</td>
<td>471</td>
</tr>
<tr>
<td>RA/BDNF 72 Hour</td>
<td>18</td>
<td>451</td>
</tr>
<tr>
<td>RA/BDNF +Collagen 72 Hour</td>
<td>17</td>
<td>493</td>
</tr>
<tr>
<td>n=60</td>
<td>n=1910</td>
<td></td>
</tr>
</tbody>
</table>

*Table 2* Indicates the number of pictures as well as the number of individual cells for each experimental condition. There were 60 total pictures, and there were a total of 1,910 individual cells. The entire set is composed of two separate differentiation experiments performed at separate times.
Figure 2 Shows in gray the expected percent for differentiated cells based on null hypothesis. The black bars indicate the actual percent differentiation in each group. Differentiated cells on plastic make up 48.5% of all differentiated cells, cells differentiated on collagen make up 51.5% of total differentiated cells. Collagen does not affect the percentage of differentiated cells.

Figure 3 Shows the percentage of cells in each sample that fully differentiated and adopted neuronal morphology. 87.6% of cells differentiated on collagen, and 90.24% of the cells differentiated on plastic, exhibited neuronal morphology.
Figure 4 Only SH-SY5Y cells treated with differentiation medium display neurite outgrowth. Each data point in the experiment is superimposed (black dots). Gray bars indicate the 95% confidence interval. N refers to the number of images used in the analysis. Images were taken from two separate differentiation experiments. For time 0 control, N=7 and neurite outgrowth was 0 um. For undifferentiated cells grown on collagen, N = 6 and neurite outgrowth was 0 um. For undifferentiated cells grown on plastic, N = 12 and neurite outgrowth was 0 um. For differentiated cells grown on collagen, N = 18 and neurite outgrowth was 59.22 ± 4.78 um. For differentiated cells grown on plastic, N = 17 and neurite outgrowth was 57.44 ± 3.32 um.
Figure 5 shows representative melt curves out of the 48 total for each primer set used in qPCR experiments. The red line in each graph indicates the threshold of fluorescence detection of the Bio Rad CFX96 Touch™ Real-Time PCR detection system. The X axis indicates temperature and the Y axis is the inverse of relative fluorescence units. RPL19, GAPDH, DAT, ChAT, SERT and NET are considered valid primers. TH is considered invalid because the main peak falls below or barely exceeds threshold value for all samples, and many samples displayed multiple peaks. Arrows indicate multiple subthreshold peaks.
Figure 6 displays the change in expression of normalization genes between all samples. The X axis includes all samples in random order including: Time 0, Negative control on collagen 72 hour, Negative control on plastic, Differentiated on plastic, and Differentiated on collagen. Ct is on the y axis. RPL19 values are plotted in grey and GAPDH in blue. Line of best fit is overlaid in the corresponding color. Variance is slightly higher in RPL19 (1.43) than GAPDH (1.13).
Figure 7 shows preliminary data from an earlier project in our lab using SH-SY5Y cells via RT-PCR with detection of gene product by agarose gel electrophoresis. Each lane is labeled with a number and the caption shows the contents of each well.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA 2-log ladder</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH T0</td>
</tr>
<tr>
<td>3</td>
<td>RPL19 T0</td>
</tr>
<tr>
<td>4</td>
<td>TH T0</td>
</tr>
<tr>
<td>5</td>
<td>DAT T0</td>
</tr>
<tr>
<td>6</td>
<td>RPL19 differentiated 72hr</td>
</tr>
<tr>
<td>7</td>
<td>TH differentiated 72hr</td>
</tr>
<tr>
<td>8</td>
<td>GAPDH differentiated 72hr</td>
</tr>
</tbody>
</table>

**Figure 7A**

**Figure 7B**
Figure 7C

DNA 2-log ladder
GAPDH T0
RPL19 T0
TH T0
DAT T0
GAPDH differentiating T24
RPL19 differentiating T24
TH differentiating T24
DAT differentiating T24
GAPDH differentiated T72
RPL19 differentiated T72
TH differentiated T72
DAT differentiated T72.
Figure 8a displays fold-change on y-axis and the experimental sample on the x-axis for DAT expression compared with Time 0 DAT expression. In all cases DAT expression decreased over 72 hours. Differentiating cells on collagen resulted in the largest decrease in expression with a fold change of 0.32. Negative control cells after 72 hours had a fold change of 0.47 and differentiated cells on plastic plates had a fold change of 0.69. Statistical analysis was performed using the $\Delta C_t$ values to conduct one-way ANOVA; no statistically-significant effect was found.

Figure 8b Shows mean $\Delta C_t$ for each condition with error bars indicating the standard error of the mean. Statistical analysis was performed using the $\Delta C_t$ values to conduct one-way ANOVA; no statistically-significant effect was found.
Figure 9a displays fold-change on y-axis and the experimental sample on the x-axis for ChAT expression compared to Time 0 ChAT expression. 72 Hour control represents a 0.82 fold-change or a decrease in gene expression. Differentiated cells on collagen represents the largest decrease in gene expression at 0.48 fold-change. Differentiated cells on plastic plates shows an upregulation of ChAT with a 1.15 fold-change.

Figure 9b Shows mean Δ Ct for each condition with error bars indicating the standard error of the mean. Statistical analysis was performed using the Δ Ct values to conduct one-way ANOVA; no statistically-significant effect was found.
Figure 10a displays fold-change on y-axis and the experimental sample on the x-axis for SERT expression compared to time-0 SERT expression. There is a decrease in expression of SERT in all cases after 72 hours. The 72-hour control shows a fold-change of 0.27, the differentiated cells on collagen show a fold change of 0.19, and the differentiated cells on plastic show a fold change of 0.63.

Figure 10b Shows mean Δ Ct for each condition with error bars indicating the standard error of the mean. Statistical analysis was performed using the Δ Ct values to conduct one-way ANOVA; no statistically-significant effect was found.
**Figure 11a** displays fold-change on y-axis and the experimental sample on the x-axis for NET expression compared to time-0 NET expression. There is a large upregulation of NET in all samples after the 72 hours. Control 72Hr cells show a fold change of 6.18, Differentiated cells on Collagen shows a fold change of 4.66, and differentiated cells on plastic shows a fold change of 5.75.

**Figure 11a** Shows mean Δ Ct for each condition with error bars indicating the standard error of the mean. Statistical analysis was performed using the Δ Ct values to conduct one-way ANOVA; no statistically-significant effect was found.
**Discussion:**

In all cases, regardless of substrate, the treatment of SH-SY5Y cells with RA and BDNF gives rise to fully differentiated neuron-like cells as defined by the extension of neurites and the presence of neuronal morphology. There is no indication that using collagen as a substrate affects the number of cells that commit to differentiation. Because there were no differentiated cells in control groups, we know that RA and BDNF induce the morphological changes indicative of differentiation, commensurate from previous findings (Encinas *et al.* 2000).

To address the noticeable clumping of the control SH-SY5Y cells after 72 hours we can look at the progression of cell density over the experiment. We see from Figure 1 that at the start of the experiment cells are sparse and not clumped, at 24 hours without RA and BDNF cells begging to appear clumped and growing together. At 72 hours we see large clumps of cells that are not differentiated. A possible explanation for this occurrence is that some of the undifferentiated cells die. A subpopulation of cells is able to survive by some intrinsic factor or association with other cells in culture. Surviving cells continue to proliferate from as an isolate which appears as a clump.

Treatment with RA and BDNF is necessary to induce neurite outgrowth. Collagen provides not only a scaffold for neurite outgrowth and a three-dimensional growth surface, but it may act as an extracellular signaling molecule by activating integrin related signaling pathways that may result in increased neurite length (Li *et al.*, 2007). While cells differentiated on a collagen substrate displayed slightly longer neurites, the difference in neurite length was not statistically significant in our experiments. It is unclear from this study what genes may be upregulated during differentiation to cause the increase in neurite length, but we can speculate that it is likely genes involved in modulation of cytoskeletal elements, as the formation of
neurites is caused by the modulation of certain cytoskeletal components (Li et al., 2007). Additionally, it is important to note that our harvesting protocol selected for only adherent cells because we aspirated off any suspended cells that were not adherent to the plate. All the cells we examined were associated with the substrate of the plate they were on. Selecting for adherent cells is a common practice with the use of the SH-SY5Y cell line because we want to examine only the cells that we know are in association with ECM molecules.

Melt curve analysis demonstrated that not every primer set used in this study was valid for qPCR. GAPDH, DAT, ChAT, SERT, and NET showed consistent melt curves in every sample with strong, single peaks. RPL19 had generally consistent melt curves, however there was much more variation in RPL19 Ct values than in GAPDH C\textsubscript{T} values between samples. TH failed to provide consistent melt curves, indicating that the TH primer is either non-functional under the qPCR protocol or generates non-specific products. The TH primer may also have been influenced by the small amount of genomic DNA contamination that we were unable to remove from samples. We decided to continue our analysis using only RPL19, GAPDH, DAT, ChAT, SERT and NET to avoid wasting reagents. Besides the TH, primer runs we had to discard the results from 4 qPCR wells with RPL19 because the C\textsubscript{T} values were more than 2 away from the mean for the triplicate. These outliers were likely a result of unequal pipetting.

When determining what gene was the most appropriate to use as our normalization gene, we chose GAPDH. There was less variance in C\textsubscript{T} value between samples, which is crucial when selecting a normalization control. The differences in RPL19 C\textsubscript{T} values between samples may be either a result of the primer binding suboptimally, or of a large variation in expression of this gene.
All of our marker genes were detectably expressed in all conditions, including undifferentiated control cells. It is possible that unidentified factors in the proliferation medium (which contains fetal bovine serum) are inducing the baseline expression of DAT, ChAT, SERT and NET. Although undifferentiated SH-SY5Y cells do not show neuronal morphology, they do express neurotransmitter transporters, whose expression is typically restricted to neuronal cells.

Because there is DAT expression without neuronal morphology in 0 hour control cells, it is clear that neuritogenesis is not necessarily linked to expression of proteins contributing to monoamine homeostasis. If anything, downregulation of DAT, SERT, and ChAT was observed in differentiated cells. These results were contrary to our expectations but represent a useful finding for the characterization of these cells: contrary to previous reports based on neuronal morphology and TH protein expression (Encinas et al., 2000). SH-SY5Y cells do not appear to exhibit a distinctly dopaminergic phenotype following sequential treatment with RA and BDNF. From these results, we cannot be certain if the presence of multiple neurotransmitter phenotypes is a result of differential expression of subpopulations of SH-SY5Y cells in culture or if it is from homogeneous expression of multiple neurotransmitter markers across the entire population. A immunohistochemical assay targeted at all of markers would go a long way toward answering this question.

The goal of this research was to determine a methodological approach for the induction of dopaminergic phenotype in SH-SY5Y cells that strikes a balance between cost and level of specific phenotypic induction, as well as to characterize the neurotransmitter phenotype, of differentiated cells. Although our differentiated cells did not adopt a specifically dopaminergic phenotype the findings are still useful, as they show that dopamine active transporter was expressed at a low level in all experimental conditions. Additionally, the results of this study
may be important for the many researchers using SH-SY5Y cells treated with RA and stably transfected with DAT in studies of drugs and diseases that affect the dopamine transporter. DAT and NET are extremely closely-related proteins, and DAT and NET are also closely related to SERT. The expression of all three monoamine transporters in these cells may prove to be a serious experimental confound in many research projects. Recently, attention has shifted towards understanding the molecular and cellular mechanisms controlling the dopamine membrane transporter in terms of transcription, placement and function at the neural synapse. This has led to the understanding of many new protein-protein interactions that involve DAT (reviewed in Sager and Torres, 2011). These interactions control the catalytic activity of DAT and the modulation of DAT at the synapse (Eriksen et al. 2010). Additionally, there have been new methods for studying DAT regulation in cells. One such example is the utilization of fluorescently tagged amphetamine molecules that react with DAT in order to observe the localization and activity of DAT under various conditions (Eriksen et al. 2010). Amphetamines target DAT, NET and to some extent SERT. As for RA /BDNF treated SH-SY5Y cells, experimental exposure to amphetamines may confound results because the cells are expressing DAT, NET, and SERT which will all react in these assays instead of controlling only for DAT. Furthermore, researchers commonly examine dopamine uptake activity in neurons using tritiated dopamine. Results from this type of assay may not be reliable in SH-SY5Y studies using this differentiation protocol, because both NET and DAT would be utilized in this reaction. Lastly, toxicity studies using 6-OHDA and MPP+ may be unreliable in RA/BDNF differentiated SH-SY5Y cells because they contain NET. The OHDA and MPP+ reagents are meant to work exclusively with DAT but can and will react with NET. Results from these assays may therefore
be suspect unless NET expression is explicitly controlled for, or removed from the cells via siRNA treatment, CRISPR, or other techniques.

These findings call in to question the validity of using RA and BDNF differentiated SH-SY5Y cells as a model for dopaminergic neurons especially in the study of Parkinson’s disease. Because these cells are expressing several neurotransmitters at the same time, they may not be a valid model for dopamine related diseases unless expression gene expression is controlled through genetic engineering techniques.

Additional future directions of this experiment are aimed at redesigning primers for tyrosine hydroxylase that will work with current qPCR methodology. Also, the fact that our cells were passaged up to 8 times before any differentiation protocol were performed presents an unknown. We cannot be certain that culturing cells in fetal bovine serum and in close proximity to one another did not start differentiation or at least affect intercellular signaling. To alleviate this confound it is important to compare the current results of baseline expression to baseline expression in freshly shipped P0 cells. However, passaging around 90% confluency is defined by the protocol we used (Encinas et al. 2000) and baseline expression of neurotransmitter markers has only been previously reported previously using microarray technology (Korecka et al, 2013), which typically does not provide robust results at the individual gene level and requires additional validation. A repetition of qPCR analysis is in store with DNase digestion of RNA templates to remove any possible genomic DNA contamination. Additionally, further replicating our PCR experimentation will increase its statistical power. Our observations of gene expression level changes in our cells should be confirmed at the protein level through Western blot analysis. Although we do have data to support transcription level it would be important to look at protein in case there is any post-translational modification in the SH-SY5Y.
Lastly, we performed a power analysis using Cohen's d which requires taking the mean difference between groups and dividing by the standard deviation. We found that in almost all of our conditions, most of the effect sizes or mean differences are below 1 standard deviation different. This indicates that the majority of our samples have a small effect size (below .5), for these samples it would require many more replication of the experiment to determine a statistically significant difference. Although we did not yield fully differentiated dopaminergic cells, we did observe widespread changes in gene expression for various neurotransmitter markers. Our cells had notable upregulation in norepinephrine markers and some of our cells experienced upregulation in cholinergic markers, consistent with the most common phenotypes found in neurons of the sympathetic ganglia. The differentiated SH-SY5Y cells also produce SERT, an unexpected result given the developmental lineage of these cells.

By characterizing the SH-SY5Y cells in terms of neurotransmitter phenotype we will be able to inform future research using the cell line as a purely dopaminergic or catecholaminergic model that there is expression other neurotransmitter phenotypes. Dysregulation of gene expression in SH-SY5Y cells both before and after differentiation may confound any results from experimentation that aims to addresses only one neurotransmitter phenotype.
References


