Biochemical characterization of proliferative and differentiated SH-SY5Y cell line as a model for Parkinson's disease.

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Abstract:

Parkinson's disease is a multifactorial neurodegenerative disease. The cellular pathology includes dopamine depletion, decrease in mitochondrial complex I enzyme activity, lysosomal glucocerebrosidase enzyme activity and glutathione levels. The SH-SY5Y human neuroblastoma cell line is one of the most widely used cell line model for Parkinson's disease. However, the consensus on its suitability as a model in its proliferative or differentiated state is lacking. In this study, we characterized and compared the biochemical processes most often studied in PD. This in proliferative and differentiated phenotypes of SH-SY5Y cells and several differences were found. Most notably, extracellular dopamine metabolism was significantly higher in differentiated SH-SY5Y. Furthermore, there was a greater variability in glutathione levels proliferative phenotype (+/- 49%) compared to differentiated (+/- 16%). Finally, enzyme activity assay revealed significant increase in the lysosomal enzyme glucocerebrosidase activity in differentiated phenotype. In contrast, our study has found similarities between the two phenotypes in mitochondrial electron transport chain activity and tyrosine hydroxylase protein expression. The results of this study demonstrate that despite coming from the same cell line, these cells possess some key differences in their biochemistry. This highlights the importance of careful characterization of relevant disease pathways to assess the suitability of cell lines, such as SH-SY5Y cells, for modelling PD or other diseases, i.e. when using the same cell line but different differentiation sates.

Abbreviations:

3-OMD, 3-O-Methyldopa; 5-HIAA, 5-hydroxyindolacetic acid; AADC, Aromatic amino acid decarboxylase;; DA, Dopaminergic; DAT, Dopamine transporter; DOPAC, 3,4-dihydroxyphenylacetic acid; ETC, Electron transport chain; GBA, Glucocerebrosidase; GSH, Glutathione; HVA, Homovanillic acid; L-DOPA, L-3,4-dihydroxyphenylalanine; MPP^+, 1-methyl-4-phenylpyridinium; PD, Parkinson's Disease; RA, Retinoic acid; TH, Tyrosine hydroxylase; TPA, 12-O-Tetradecanoylphorbol-13-acetate; TPH, Tryptophan hydroxylase.

1. Introduction:

Parkinson's disease (PD) is a multifactorial progressive neurodegenerative disease that affects about 1% of people over the age of 65. Diagnosis is based on the clinical presentation of movement abnormalities that include resting tremor, postural instabilities, bradykinesia, and rigidity which are a consequence of dysregulated dopamine system. Patients also suffer from non-movement related symptoms that affect their sleep, mood and digestive health; behaviours regulated by the serotonin system (Politis et al., 2015). Pathophysiology of the disease centre around the presence of Lewy body inclusions and death of the majority of dopaminergic (DA) neurons of the *substantia nigra* area of the brain. Research in post-mortem PD brain tissue samples has revealed the perturbation of several biochemical pathways. These include decreased activity of mitochondrial electron transport chain (ETC) complex I (Schapira et al., 1990), decreased protein expression and activity of lysosomal enzyme glucocerebrosidase (GBA, Gegg *et al.* 2012) and decreased levels of the antioxidant glutathione (GSH, Riederer *et al.* 1989). Current available therapies aim for dopamine replacement, for example by providing *L*-3,4-dihydroxyphenylalanine (*L*-DOPA), but this is not curative and is associated with many undesirable side-effects.

Dopamine and serotonin are important neurotransmitters in the central nervous system (CNS). The classical pathway of dopamine biosynthesis takes place in the cytosol of DA neurons. It starts with the hydroxylation of *L*-tyrosine to produce *L*-DOPA by the enzyme tyrosine hydroxylase (TH). This first step is the rate limiting step of dopamine biosynthesis and is tightly regulated. In the cell, dopamine is stored in acidic vesicles before release into the synaptic cleft following neuronal excitation. Dopamine signal is transmitted by interacting with pre- and post-synaptic dopamine receptors. Dopamine signal is terminated via uptake either by DA neurons through the action of dopamine transporters (DAT), or by glial cells for degradation. Dopamine degradation results in production of the main metabolites in the CNS, 3,4-dihydroxyphenylacetic acid and homovanillic acid (DOPAC and HVA, Meiser *et al.* 2013). Serotonin biosynthesis and degradation shares similarity with dopamine metabolism as some enzymes are common to the metabolism of both. The principal serotonin metabolite in the brain is 5-hydroxyindolacetic acid (5-HIAA, Hyland 2008).

Appropriate models are crucial for any scientific study. PD exhibits a multifactorial pathology that involves different cellular processes including but not limited to α-synuclein aggregation, dopamine and serotonin metabolic dysregulation, altered glutathione status, mitochondrial dysfunction and autophagy-lysosomal pathway dysregulation (Toulorge et al., 2016).

Concerning model systems, the SH-SY5Y neuroblastoma cell line is one of the most widely used cell models in PD research (Chutna et al., 2014; Hasegawa et al., 2004; Meka et al., 2015). The assumption that SH-SY5Y cells can be dopaminergic, and therefore suitable for PD research, is based on their expression of TH, the rate limiting step in dopamine synthesis, and DAT (Xicoy et al., 2017). However, measurement of intracellular dopamine and serotonin metabolites can provide a functional readout of their metabolism and therefore assess their suitability as a model. In addition, CSF analysis of neurotransmitter metabolism is a crucial clinical tool to diagnose and monitor disorders that affect the CNS (Hyland, 2008). Measurement and characterisation of extracellular neurotransmitter metabolism in CNS disease models can provide a good analogy. Furthermore, SH-SY5Y cells have been used as a model to study PD related mitochondrial dysfunction. For example, they have been used to study the effect of the neurotoxins 1-methyl-4-phenylpyridinium (MPP^+), 6-hydroxydopamine and rotenone on mitochondrial bioenergetics and dynamics (Giordano et al., 2012; Wang et al., 2011), the effect of dopamine oxidation on mitochondrial function (Biosa et al., 2018) and effect of mitochondrial inhibition on dopamine and serotonin metabolism (de la Fuente et al., 2017). Additionally, SH-SY5Y cells were used to study PD related lysosomal dysfunction in terms of effect of lysosomal GBA inhibition on dopamine and serotonin metabolism (de la Fuente et al., 2017), effect of heterozygous GBA mutations on mitochondrial function (Li et al., 2019) and effect of GBA inhibition on mitochondrial function and oxidative stress (Cleeter et al., 2013). Finally, SH-SY5Y cells have been employed to assess the glutathione system in response to dopamine and L-DOPA induced oxidative stress (Allen et al., 2013) and effect of the neurotoxin rotenone on GSH levels (Watabe and Nakaki, 2008).

Despite this, there is controversy in the literature whether SH-SY5Y should be used in the proliferative state or whether differentiation is needed (Hong-rong et al., 2010; Kovalevich and Langford, 2013; Luchtman and Song, 2010; Xicoy et al., 2017). Proliferative SH-SY5Y cells are continuously dividing, giving rise to a cell population that might not be completely homogeneous, and are, by their proliferative state, dissimilar to adult neurons which are post-mitotic. Constant proliferation increases the rate of DNA replication error therefore higher passage number populations may not behave in the same way as lower passage number populations. Differentiation of SH-SY5Y cells confers morphological, biochemical, ultrastructural, and electrophysiological characteristics that are reported to better resemble mature neurons (Xicoy et al., 2017). Differentiation halts proliferation, synchronises the cell cycle and produces a more homogenous cell

population (Hong-rong et al., 2010). Most notably, it has been reported that differentiated cells have increased expression of TH (Ballaz et al., 2013; Looyenga et al., 2013; Presgraves et al., 2004) and DAT (Presgraves et al., 2004). Retinoic acid/tetradecanoyl phorbol acetate (RA/TPA) differentiation of SH-SY5Y cells is reported to drive cells into a more dopaminergic lineage (Presgraves et al., 2004). Previous research comparing proliferative and differentiated SH-SY5Y cell line suitability as a model have shown that SH-SY5Y cell differentiation increases TH expression, renders cells less susceptible to MPP$^+$, and that the electrophysiology of differentiated SH-SY5Y cells better resemble mature neurons (Khwanraj et al., 2015; Şahin et al., 2021). Similar arguments surrounding the need for differentiation have been drawn comparing proliferative and differentiated Lund Human Mesencephalic (LUHMES) neuronal cell line, where differentiated LUHMES were found to exhibit a proteomic profile that better resemble primary midbrain neurons compared to its proliferative phenotype (Tüshaus et al., 2021).

Despite these assumptions on the basis of TH expression, dopamine and serotonin metabolism, mitochondrial function, lysosomal GBA and glutathione levels have not been fully characterised in proliferative and differentiated SH-SY5Y cells. Therefore, the aim of this study was to explore these biochemical pathways in proliferative and differentiated SH-SY5Y cells to assess their suitability for research into PD and related diseases.

2. Materials and methods:

2.1. Materials:

The following materials were acquired from Sigma Aldrich (Poole, UK); Dulbecco's phosphate buffered saline, 3,4-dihydroxyphenylacetic acid, 5-hydroxyindole-3-acetic acid, homovanillic acid, dopamine hydrochloride, 3-methyl-L-tyrosine monohydrate, 3,4-dihydroxy-L-phenylalanine, 1-octanesulfonic acid sodium salt, EDTA disodium, sodium acetate trihydrate, citric acid monohydrate, retinoic acid and hydrochloric acid. HPLC grade methanol, 0.25% Trypsin-EDTA, heat inactivated foetal bovine serum (FBS), Dulbeccco's modified Eagle's medium/Ham's F12 1:1 nutrient mix (DMEM/F-12), DMEM/F-12 phenol-red free media, 12-O-tetradecanoylphorbol-13-acetate, and GlutaMAX™ L-glutamine solution were purchased from Thermo Fisher Scientific (Loughborough, UK),

2.2. Cell culture:

SH-SY5Y cells (ECACC, Cat# 94030304, RRID: CVCL_0019) were cultured in DMEM/F12 media + 2 mM GlutaMAX™ L-glutamine supplemented with 10% FBS (10%FBS). Cells were cultured at 37°C and 5% CO2. Passages between 18 and 24 were used, and media were changed every 3 days.

For differentiation of SH-SY5Y cells, the differentiation protocol was as described by Presgraves et al. (2004). SH-SY5Y cells were seeded at 10^4 cells/cm^2 in 10% FBS medium (differentiation day 0). The following day cells were treated in the dark with 10 μM retinoic acid in DMSO for 3 days followed by 80 nM 12-O-Tetradecanoylphorbol-13-acetate (TPA) for 3 days in reduced serum media (1% FBS). Experiments were performed on day 7.

For proliferative cells, cells were seeded on day 0 at 4 x 10^3 cells/cm^2 in 10% FBS medium. Media were changed every 3 days.

2.3. L-DOPA treatment and sample preparation:

SH-SY5Y cells were treated for 1 hour with 100 μM L-DOPA in phenol-red free DMEM/F12 supplemented with 10% of FBS, 2 mM GlutaMAX™ L-glutamine. For extracellular neurometabolites, a sample of cell culture medium was diluted 1:1 in ice cold 0.8 M perchloric acid (PCA) and incubated on ice in the dark for 10 minutes. Sample was then centrifuged at 12000g for 5 minutes at 4 °C and the supernatant was analysed by HPLC with electrochemical detection (HPLC-ECD). For intracellular neurometabolites, cells were collected, washed once with PBS then suspended in 1 ml ice cold lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 320 mM sucrose in HPLC grade water). Cell lysate was diluted

1:1 in ice cold 0.8 M PCA, centrifuged at 12000g for 5 minutes at 4 °C and supernatant was analysed by HPLC-ECD. Protein concentration was determined using a Bradford assay.

2.4. Western blot for tyrosine hydroxylase:

SH-SY5Y cells were collected and washed with PBS then suspended in RIPA buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin) + protease inhibitor cocktail and incubated on ice for 5 minutes. Lysate was centrifuged at 12000g for 15 minutes and supernatant was collected. Protein concentration was determined by a BCA assay according to manufacturer's instructions. 10 μg protein/sample was separated on a 4–20% polyacrylamide gels then electro-transferred using Bio Rad Trans-Blot turbo™ transfer pack and the Bio Rad Trans-Blot TURBO™ transfer system set at 25 V, 2.5 A for 7 minutes. Following blocking the membrane for 1 hour in 5% milk-TBST (TBST: 0.5 L TBS (1 Tris-HCl tablets + MilliQ water) + 0.5 ml Tween-20), membrane were probed with a primary anti-TH antibody developed in rabbit (Sigma Aldrich, #T8700, RRID:AB_1080430, 1:1000) or horseradish peroxidase conjugated anti-GAPDH antibody (Cell Signaling Tech, #3683, RRID:AB_1642205, 1:1000) in 1% milk-TBST overnight. Membranes were then washed with TBST then incubated with anti-rabbit secondary antibody for 1 hour (1:3000) in 1% milk-TBST. Membrane was then washed with TBST, cut in half, and imaged using Bio Rad Clarity Western ECL solution and Bio Rad ChemiDOC MP imaging system. Protein molecular weight ladder was used to identify bands and bands were quantified using ImageJ software. GADPH expression was used as control.

2.5. HPLC measurement of dopamine and monoamine metabolites:

Quantification of neurometabolites (DOPAC, 3-OMD, 5-HIAA, HVA and dopamine) was carried out using reverse phase HPLC and an electrochemical detector following a method by de la Fuente et al. (2017). The mobile phase (flow rate 1.5ml/min) contained 16% methanol, 20 mM sodium acetate trihydrate, 12.5 mM citric acid monohydrate, 3.35 mM 1-octanesulfonic acid, 0.1 mM EDTA disodium and adjusted to pH 3.45 with 12 M hydrochloric acid (HCl). The stationary phase was maintained at 27 °C. The detector electrode was set at 450 mV and screening electrode at 50 mV. 50 μl of sample was injected and calculated against a 500 nM external standard solution of the 5 compounds of interest made in HPLC grade water acidified with 12 M HCl. Peak areas were quantified with EZChrom Elite™ chromatography data system software, version 3.1.7 (JASCO UK Ltd., Great Dunmow, UK).

2.6. HPLC measurement of glutathione:

Glutathione levels in cell lysate was measure by a method adapted from (Allen et al., 2013). GSH was extracted from SH-SY5Y cells using 15 mM O-phosphoric acid and separated on a C18HS 250 mm x 4.6 mm reverse-phase HPLC column (Kromatek, Dunmow, UK) maintained at 35 °C, with flow rate 0.5 ml/min using 15 mM O-phosphoric acid as mobile phase. GSH was detected using an electrochemical detector with scanning electrode set at 50 mV and detector electrode set at 550 mV. 5 μM GSH was used as a standard to determine concentration.

2.7. Enzyme activity:

For mitochondrial electron transport chain activity, sample was prepared by suspension of SH-SY5Y cells in lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 320 mM sucrose in HPLC grade water) and sonicated on ice. Enzyme activity was measured spectophotometrically as previously described (Hargreaves *et al.* 2007) and expressed as ratio to citrate synthase activity. Briefly, complex I was measured as rotenone-sensitive NADH-oxidation at 340nm with ubiquinone$_1$ as electron acceptor, complex II-III as antimycin A –sensitive succinate-cytochrome c reductase at 550nm, complex IV as reoxidation of reduced cytochrome c at 550nm (as a first-order rate constant), and citrate synthase as oxaloacetate-dependent CoASH release from acetyl-CoA, with CoASH release measured using 5,5'-dithio-bis-(2-nitrobenzoic acid) at 412nm.

For lysosomal GBA, a protocol adapted from (Gegg et al., 2012) was used. Sample was prepared by collecting SH-SY5Y cells in PBS and sonicating on ice. For GBA, in a 96 well plate, 10 μl of sample, 15 μl 148.8 mM sodium taurocholate and 25 μl of McIlvaine citrate-phosphate buffer pH 5.4 were added. At timed intervals, 50 μl of 10 mM 4-Methylumbelliferyl β-D-glucuronide (4-MUG, substrate) was added to all wells. Plate was incubated at 37 °C for 1 hour then 110 μl 2.5 M glycine (pH 10.4) solution was added to stop the reaction. For a standard, 200 μl of 5 μM 4-Methylumbelliferone was added with 100 μl of stop solution. Fluorescence was measured at 340nm excitation and 465nm emission.

2.8. Statistical analysis:

Results are presented as mean ± standard error of the mean (SEM). Individual comparisons of means were made using unpaired Student's t-test when variance was equal. An unpaired Student's t-test with Welch's correction was used when variance was unequal. An F-test was used to check for

variance. GraphPad Prism statistical software was used (Version 8, GraphPad Software INC. CA, USA). $p < 0.05$ was considered significant.

3. Results:

3.1. SH-SY5Y differentiation:

Proliferative SH-SY5Y cells have a morphology that resembles that of mesencephalic cells and are continually proliferating. As expected, after differentiating by sequential treatment with RA and TPA for 7 days, cells stopped proliferating, became a stable population, and acquired obvious morphological changes. They became more neuronal-like in cell shape; the cell body became polarised, neurites were extended, and neurite branching was observed. In addition, differentiated SH-SY5Y cells appeared to cluster together and extend their neurites between different clusters in what appeared to be making connections.

3.2. Detection of tyrosine hydroxylase protein expression:

TH is a homo-tetramer protein where each monomer has an approximate molecular weight of 60 kDa (Szigetvari et al., 2019). Immunoblotting using a specific antibody against TH monomers in proliferative and differentiated cells showed a band at approximately 60 kDa (**Figure 1**). The band identity was confirmed by comparison with iPSC dopaminergic neurons (Gift from Professor Manju Kurian UCL, London, UK). Using band densitometry to quantify the level of protein expression, and after normalisation to GADPH band density, level of TH expression in proliferative (TH/GAPDH 0.24) and differentiated (TH/GAPDH 0.23) cells was similar.

3.3. Intracellular and Extracellular Dopamine and monoamine metabolites

PD is a disease associated with dysregulated dopamine and serotonin metabolism; therefore, characterisation of their metabolism is important for consideration of any disease model.

Under basal conditions, intracellular and extracellular dopamine and its metabolites were not detectable in either proliferative or differentiated SH-SY5Y cells. In contrast, extracellular 5-HIAA (the main serotonin metabolite) was readily detectable and similar in proliferative and differentiated cells (3507 ± 411 pmol/mg protein in proliferative, and 3561 ± 968 pmol/mg protein in differentiated).

Following treatment for one hour with 100 µM L-DOPA, extracellular dopamine and serotonin metabolites were detected. There was a significant difference in the level of extracellular dopamine between proliferative and differentiated cells (18984 ± 4306 pmol/mg protein in proliferative 4046 ± 384 pmol/mg protein in differentiated. $p = 0.01$, **Figure 2** A). DOPAC levels were lower in differentiated cells compared to proliferative cells, 4363 ± 1856 pmol/mg protein and 1582 ± 293 pmol/mg protein ($p = 0.11$), respectively. Levels of 3-OMD, 5-HIAA and HVA were similar between the

two cellular phenotypes (3-OMD was 10169 ± 2048 pmol/mg protein in proliferative and 10275 ± 1590 pmol/mg protein in differentiated, p= 0.96. 5-HIAA was 3523 ± 670 pmol/mg protein in proliferative and 3790 ± 818 pmol/mg protein in differentiated, p=0.81. HVA was 890 ± 330 pmol/mg protein in proliferative and 1178 ± 307 pmol/mg protein in differentiated, p= 0.53, **Figure 2** A). Dopamine turnover rate was evaluated by determining the ratio of the metabolites, DOPAC + HVA to dopamine. A significantly greater (2.5-folds) turnover rate was observed in the differentiated cells (Dopamine turnover was 0.25 ± 0.04 in proliferative and 0.65 ± 0.08 in differentiated. p= 0.0012, **Figure 2** B).

Intracellularly however, only dopamine and its metabolites were detected. Dopamine levels were higher in proliferative cells compared to differentiated, 3967 ± 865 pmol/mg protein and 2211 ± 312 pmol/mg protein, respectively (p= 0.0946). Levels of DOPAC and 3-OMD were slightly lower in differentiated but the finding was not significant (DOPAC was 1356 ± 327 pmol/mg protein in proliferative and 823 ± 205 pmol/mg protein in differentiated, p= 0.17. 3-OMD was 1379 ± 342 pmol/mg protein in proliferative and 681 ± 165 pmol/mg protein in differentiated, p=0.08, **Figure 2** C). Because HVA was not detectable intracellularly, dopamine turnover was calculated as ratio of DOPAC to dopamine. In contrast to extracellular levels, intracellular dopamine turnover was comparable between the two cell phenotypes (0.32 ± 0.02 in proliferative cells and 0.35 ± 0.04 in differentiated cells, p= 0.65, **Figure 2** D).

3.4. Mitochondrial citrate synthase and respiratory chain enzyme activities:

Reduced activity of the mitochondrial ETC complex I and IV are associated with PD pathology (Holper et al., 2019; Schapira et al., 1990). Inhibition of ETC complex I with rotenone induces changes in dopamine and serotonin metabolism (de la Fuente et al., 2017). Therefore, characterization of mitochondrial ETC complex activities   is relevant when considering a cellular model for PD. Citrate synthase (CS) enzyme activity is reported to correlate with mitochondrial content and can change after differentiation (Larsen et al., 2012; Schneider et al., 2012). In this study, an approximate 20% decrease in CS activity was observed following differentiation (**Table 1**) In view of this and to account for changes in mitochondrial enrichment, respiratory chain enzyme results were also expressed as ratio to CS. Activity of ETC complex I was found to be comparable between the two phenotypes after normalisation to CS activity (0.08 ± 0.009 for proliferative and 0.08 ± 0.007 for differentiated). ETC complex II-III activity was also found to be comparable between the two phenotypes when ratioed

to CS activity. ETC Complex IV, however, was marginally higher in differentiated cells albeit the result was not statistically significant (**Table 1**).

3.5. Lysosomal glucocerebrosidase:

Several lines of evidence have associated decrease in the activity the lysosomal enzyme, GBA, with PD pathology. Therefore, GBA activity was measured and compared between the two phenotypes. A significant increase in GBA was observed in differentiated cells (**Figure 3**, p = 0.01).

3.6. Glutathione levels:

Oxidative stress is considered a key player in PD pathology. GSH is a major antioxidant in the cell, which protects against oxidative stress (Place and Smeyne, 2013). Decreased levels of GSH have been documented in PD (Riederer et al., 1989). Therefore, GSH levels were measured and compared between the two cellular phenotypes. Although the mean GSH levels was found to be lower in differentiated cells (16.8 nmol/mg compared to 14.9 nmol/mg in proliferative), the results were not statistically significant (**Figure 4**). Interestingly however, looking at individual measurements, levels of GSH varied considerably in proliferative cells, with a 49% coefficient of variation compared to 16% in differentiated cells (f-value = 0.0004, **Figure 4**).

4. Discussion:

The SH-SY5Y cell line has been extensively used in PD research, a disease exhibiting multifactorial pathology affecting several biochemical pathways in the neuron. Mitochondrial and lysosomal dysfunctions have been associated with disease pathology. Cellular redox state and neurotransmitter imbalances have been documented (Toulorge et al., 2016). Data presented here suggests that SH-SY5Y cells could provide a good model for affected dopaminergic neurons in PD due to the absence of dopamine production under basal conditions. This observation is in line with a previous finding by de la Fuente and colleagues (2017) using proliferative SH-SY5Y where they demonstrated the requirement for *L*-DOPA supplementation for detection of dopamine and its metabolites. However, others have documented basal dopamine in proliferative SH-SY5Y cells following over-expression of TH (Prasuhn *et al.* 2018) and stimulation with KCL or ATP (Ganapathy et al., 2016). Taken together, these cells appear to recapitulate what is seen in diseased cells in PD, whereby *L*-DOPA, the gold standard therapy, is required for replenishing dopamine levels in the brain. This finding prompts the question of the reason behind inability to detect dopamine. Because dopamine and its metabolites are readily detectable following *L*-DOPA treatment and 5-HIAA is also detectable, this suggests that steps catalysed by the enzyme aromatic amino acid decarboxylase are in place. This therefore suggests that the failure to detect dopamine/dopamine metabolites may lay with the first and rate limiting step of dopamine synthesis, conversion of L-tyrosine to *L*-DOPA via TH. However, the presence of TH protein was confirmed using western blot (**Figure 1**). Both cellular phenotypes appear to express comparable levels of TH, which is similar to the findings of Korecka *et al.* (2013) and colleagues following RA differentiation of SH-SY5Y cells. In addition, presence of its cofactor, tetrahydrobiopterin (BH$_4$), can be deduced from the functional basal serotonin metabolism which also requires BH$_4$ as cofactor (**Figure 2**). Furthermore, cofactor competition between TH and tryptophan hydroxylase (TPH) for BH$_4$ can be dismissed because the Michaelis constant (K$_m$ value) of TPH for BH$_4$ is higher than that of TH (315 ± 37 µM and 5.6 ± 1.0 µM, respectively, Windahl *et al.* 2009; Briggs *et al.* 2013). Finally, cells are grown in cell culture medium containing *L*-tyrosine, iron, and ascorbate to support TH function and additional *L*-tyrosine supplementation did not drive dopamine synthesis (data not shown). This prompts several speculations. Firstly, dopamine could be synthesized but at a level below the detection limits of the HPLC-ECD method used in this study (< 50 pmol). Secondly, TH activity is tightly regulated via phosphorylation/dephosphorylation by several protein kinases such as protein kinase A and C (PKA and PKC, Dunkley *et al.* 2004). Therefore,

induction of upstream signalling pathways may be required for TH activity, as an additional layer of regulation of dopamine metabolism. Finally, because the serotonin metabolite 5-HIAA was readily detectable in proliferative and differentiated SH-SY5Y cells under basal condition, the cells may have a stronger serotonergic phenotype. Following supplementation with *L*-DOPA, differences in dopamine metabolism were observed between the two cellular phenotypes. Most notable was the decreased variability between individual measurements of extracellular dopamine (coefficient of variance for was 64% for proliferative and 30% for differentiated, f-value = 7.9×10^{-8}, **Figure 2** A) and the increase in dopamine turnover in differentiated cells (**Figure 2** B). Differentiation induces several signalling pathways involved in growth and survival, leading to induction of relevant enzymes to the cell type (Hong-rong et al., 2010). Enzymes implicated in neurotransmitter metabolism, regulation and transmission are important for neuronal cells. It has been shown that RA differentiation increased activity of AADC in neuroblastoma cell lines (Ikeda et al., 1994). Presgraves and colleagues (2004) have shown that RA and TPA differentiation results in an increase expression of TH, DAT and dopamine receptors D_2 and D_3, proteins that are central to dopamine metabolism and regulation (Presgraves et al., 2004). These findings may suggest that differentiated cells differ in the activity of the enzymes implicated in dopamine metabolism and/or regulation.

The observed increase in the lysosomal GBA following differentiation (**Figure 3**) might be a result of induction of the sphingolipid pathway, that is an important pathway for providing structural precursors for cell membranes and neuronal myelin sheath (Rao et al., 2013). Differentiation confers extensive morphological changes to SH-SY5Y cells as a result of cellular polarisation and extension and branching of neurites, requiring synthesis of cell membrane constituents like ceramides and sphingomyelins. The induction of sphingolipid pathway seen in differentiated cells may enhance their ability to perform functions that are characteristic of mature neurons. Cellular polarisation and axon myelination are important for neurons. In addition, the membrane is a dynamic entity where its components are constantly regulated and fine-tuned to facilitate different function. In neurons, this fluidity is explicitly important for signal transduction and neurotransmission (Mencarelli and Martinez-Martinez, 2013).

As a marker for oxidative stress, a condition strongly associated with PD pathology, GSH levels are an important factor to consider when modelling the disease. The cellular redox state is represented by a balance in reduced/oxidized GSH molecules. GSH, inherently, is an unstable molecule so metabolic regulation is important to preserve this balance. Looking at individual

measurements of basal GSH, the levels were highly variable in proliferative cells compared to differentiated (**Figure 4**). Similar variability was also observed with individual measurements of dopamine (**Figure 2**). Proliferative cells are constantly proliferating; lacking synchrony in cell cycle, representing a non-homogenous cell population. The variance observed in measurements may reflect this heterogeneity. Concerning oxidative stress and the potential generation of deleterious oxidising species, future studies should also look at potential sources of reactive oxygen species and effects of differentiation, e.g. NADPH oxidase.

Decreased mitochondrial complex I activity is strongly associated with PD pathology. Loss of complex I activity have been documented in brain tissue samples from PD patients and rotenone and MPTP, potent complex I inhibitors, are routinely used to create *in vivo* and *in vitro* models of the disease (Jagmag et al., 2016; Schapira et al., 1990). Therefore, characterisation of mitochondrial electron transport chain activity is of interest when considering a disease model. In contrast to the differences found in lysosomal enzyme activities and redox capacity, mitochondrial complexes activities were similar between proliferative and differentiated SH-SY5Y cells. The marginal, although not significant, change in complex IV activity ratioed to citrate synthase between proliferative and differentiated SH-SY5Y cells observed may reflect an increase in complex IV expression similar to what has been seen previously following RA differentiation of SH-SY5Y cells (Schneider et al., 2012) (**Table 1**). The changes in citrate synthase activity may reflect the changes in mitochondrial content and bioenergetics (Wanet et al., 2015) therefore, comparing the ratio of mitochondrial complexes activity to citrate synthase is imperative. Similar changes were reported previously for SH-SY5Y cells, murine mesenchymal cells, myoblasts and adipose cells (Forni et al., 2016; Kraft et al., 2006; Moyes et al., 1997; Murholm et al., 2009; Schneider et al., 2012). Further work is now required to evaluate further the functional significances of these changes, e.g. downstream effects relating to any potential changes to the stoichiometry of the mitochondrial respiratory chain, i.e. with regards to parameters such as reactive oxygen species generation. Furthermore, complex IV subunit analysis may provide additional insight into the effects of differentiation. A similar approach should also be considered for lysosomal enzymes such as GBA.

Although in this work, we have focussed on the key biochemical pathways that have been implicated in the pathogenesis of PD, further work should explore the effects on the signalling pathways and mitochondrial dynamics.

In conclusion, in this study we have reported key similarities and significant differences between proliferative and differentiated SH-SY5Y cells. These in biochemical pathways that have been implicated in the pathogenesis of PD, i.e dopamine, mitochondrial, lysosomal and glutathione metabolism. Furthermore, we have demonstrated that these cells are inherently dopamine deficient and hence it can be argued that they resemble dopamine deficiency state associated with PD. Careful attention to the data reported here should be made when considering the use and phenotype of this cell line for research into conditions such as PD.

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Enzyme	nmol/min/mg protein		Ratio to Citrate Synthase		
	Proliferative	Differentiated	Proliferative	Differentiated	Significance
Citrate Synthase	157 ± 11	125 ± 9	----	----	p = 0.04
Complex I	12 ± 2	9.4 ± 1	0.08 ± 0.009	0.08 ± 0.007	ns
Complex II-III	16 ± 2	13 ± 2.5	0.096 ± 0.01	0.093 ± 0.01	ns
Complex IV	3 ± 0.5	3.6 ± 0.8	0.019 ± 0.003	0.026 ± 0.005	ns

Table 1 Enzyme activity of mitochondrial electron transport chain complexes in proliferative and differentiated SH-SY5Y cells. Activity is expressed on a protein baseline and as ratio to citrate synthase enzyme activity. Results represent mean ± SEM of 9-17 independent cell culture preparations. Unpaired Student's t test was used to test for significance. ns: not significant.
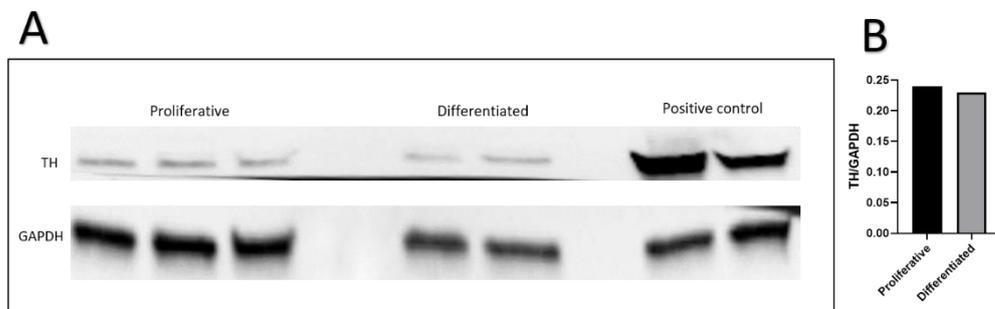
Figure 1 Immunoblot for tyrosine hydroxylase expression in proliferative and differentiated SH-SY5Y cells. iPSC dopaminergic neurons were used as a positive control for TH. GAPDH was used as a loading control. A) Representative blot from 2 independent cell culture preparations. B) Densitometry of the ratio of TH to GAPDH. TH: tyrosine hydroxylase.
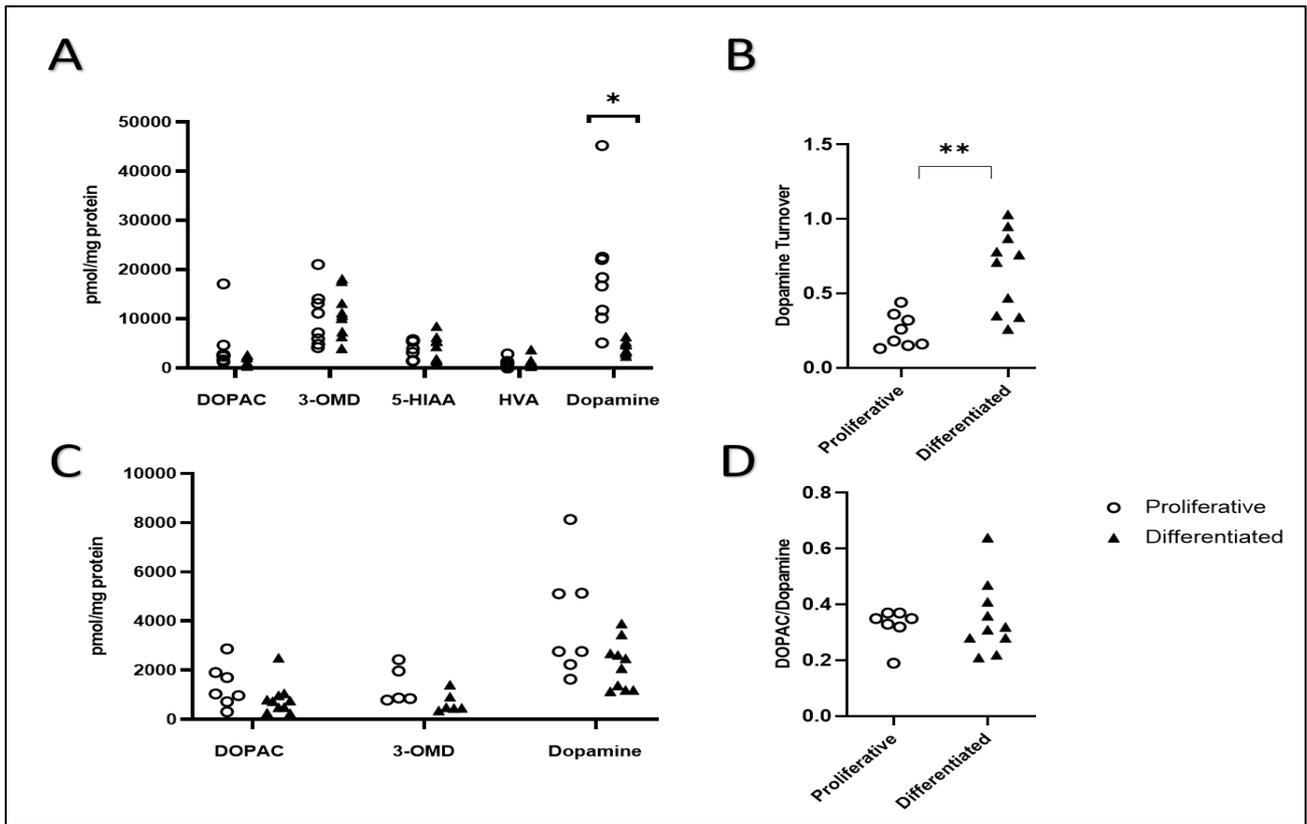
Figure 2 HPLC detection of extracellular and intracellular dopamine and serotonin metabolites in proliferative and differentiated SH-SY5Y cells. (A) extracellular metabolites of dopamine and serotonin metabolites following treatment for 1 hour with 100 µM L-DOPA (p = 0.01). **(B)** Extracellular dopamine turnover rate; calculated by ratio of dopamine metabolites DOPAC and HVA to dopamine (p = 0.0012). **(C)** Intracellular metabolites of dopamine following treatment for 1 hour with 100 µM L-DOPA. **(D)** Ratio of intracellular DOPAC to dopamine. Results are presented as mean ± SEM of 8-10 independent cell culture preparations. Unpaired Student's t-test with or without Welch's correction was used to determine significance.
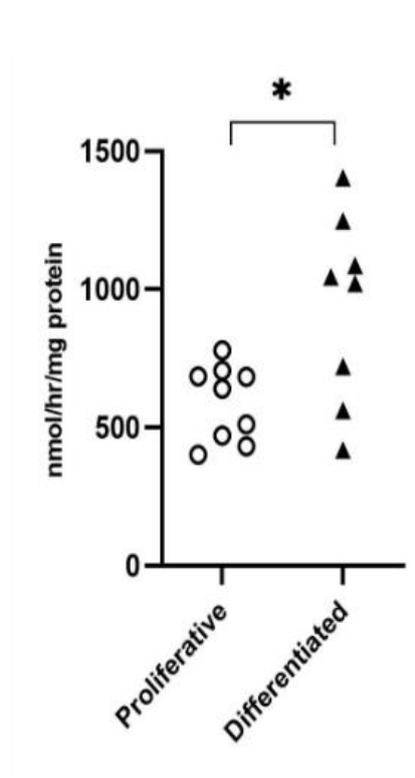
Figure 3 Enzyme activity of lysosomal glucocerebrosidase in proliferative and differentiated SH-SY5Y cells. In proliferative cells GBA activity was 668 ± 36 nmol/hr/mg protein while in differentiated 1088 ± 94 nmol/hr/mg protein (p = 0.01). Results expressed as mean ± SEM of 5-9 independent cell culture preparations. Unpaired Student's t test was used to test for significance.
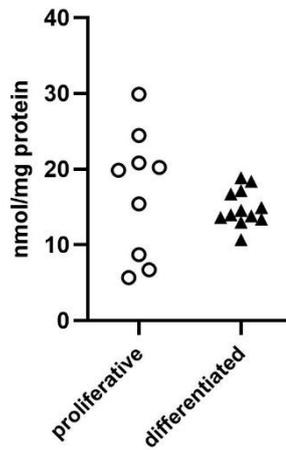
Figure 4 Glutathione levels in proliferative and differentiated SH-SY5Y cells. In proliferative cells levels of glutathione were 17 ± 3 nmol/mg protein while in differentiated 15 ± 0.7 nmol/mg protein. Results expressed as mean ± SEM of 9-12 independent cell culture preparations. Unpaired student t-test with Welch's correction was used to test for significance.