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Research Article

An Inhibited Dopamine Synthesizing Cell Model of AADC Deficiency

Melati Khalid[1,*] and Mohamad Aris Mohd Moklas[2]

[1] *Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, UPM Serdang, 43400 Serdang, Selangor, Malaysia.*
[2] *Department of Human Anatomy, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, UPM Serdang, 43400 Serdang, Selangor, Malaysia.*

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Abstract

Introduction: Aromatic L-amino acid decarboxylase deficiency (AADC) is a rare autosomal recessive pediatric neurotransmitter disease. To date it remains poorly understood mainly due to an absence of a disease model. The dopaminergic neuroblastoma cell SH-SY5Y was chosen to develop our AADC deficiency model. These cells are not native dopamine synthesizers. **Objective:** To develop a dopamine-producing cellular model of AADC deficiency using SH-SY5Y neuroblastoma cells. **Methods:** Dopamine pathway proteins were identified with Western Blotting. Dopaminergic differentiation was attempted using all-trans retinoic acid (ATRA) with dopamine detection via HPLC-ECD post alumina extraction. Treatment with L-DOPA provided SH-SY5Y with excess precursor. RT-PCR was used to determine the expression of markers of mature neurons. **Results:** Western Blot screening identified AADC, dopamine β-hydroxylase and tyrosine hyrdoxylase proteins, indicative of a dopaminergic pathway. ATRA was unsuccessful in producing dopamine from the cells. L-DOPA treatment however, generated dopamine first visible as a HPLC-ECD peak 30 minutes post-incubation. Prior to this, SH-SY5Y dopamine synthesis from L-DOPA has never been documented. This de novo synthesis is then inhibited using benserazide to form our AADC deficiency cell model. RT-PCR showed that SH-SY5Y cells express markers of mature neurons in its 'native' state and is not affected by L-DOPA and benserazide treatment. This cell model will potentially benefit many areas of AADC deficiency research. **Conclusion:** SH-SY5Y cells produced HPLC-ECD measureable amounts of dopamine with the addition of L-DOPA. Our model of AADC deficiency is generated by quelling the dopamine production with Benserazide.

Keywords: AADC deficiency; dopamine; SH-SY5Y; retinoic acid; L-DOPA

1.0 Introduction

AADC is a rare and relatively recently identified pediatric neurotransmitter disease affecting less than 200 children worldwide. Diagnosis is via identifying cerebrospinal fluid abnormalities. These are low levels of the final breakdown metabolites of dopamine and serotonin, homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) respectively; elevated dopamine and serotonin precursors L-DOPA and 5-hydroxytryptophan (5-HTP) respectively and 3-ortho-methyldopa (3-O-MD), the end-product of L-DOPA alternative breakdown pathway in the absence of AADC (Swoboda et al., 2003) (1). Upon primary findings, plasma AADC enzyme level analysis and identification of AADC gene mutation allows confirmation of the disorder. Additionally, enzyme level assays of the parents will reveal their heterozygote carrier status.

The absence of the AADC enzyme gives rise to a movement disorder with a complex array of symptoms, autonomic dysregulation being a prominent one. This include chronic nasal congestion, temperature instability (Lin 1980) (2) ptosis (droopy eyelids), excessive diaphoresis (excessive sweating), (Pons et al., 2004) (3) and Allen et al., 2009 (4)), irritability and difficulty initiating and maintaining sleep or excessive sleep (Swoboda et al., 2003) (1) presumably due to serotonin deficiency. Serotonin plays a role in lowering arousal and facilitating sleep (Voong & Eriksson, 1992) (5). It is also the precursor of melatonin, a key enzyme of the circadian rhythm. There is also a tendency for developing hypoglycemia which is thought to be caused by the relative lack of catecholamines to act as anti-insulinergic hormones (Korenke et al., 1997) (6). Emotional lability in the form of either euphoric or dysphoric mood is often observed and is believed to be due to serotonin deficiency (Saxena, 1995) (7). The most defining neurological sign is the occurrence of intermitten dystonia with or without eye movement abnormalities, also known as oculogyric crises. Dystonic episodes are resolved with sleep. The onset of dystonic oculogyric episodes is usually between the age of 1 and 6 months.

To date, AADC deficiency aetiology remains poorly understood. A cell model may offer insights on therapeutic management of this disease. For example, the ability of various medications to increase dopamine may be assessed using a cell model of the disease. This is important given that current strategies rely on adult anti-Parkinson's drugs which does not address the root cause for dopamine deficiency in AADC. Hence, their minimal benefits come with wide-ranging, serious side effects. Potential new therapies may also be screened using this cell culture.

***** *Correspondence: Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, UPM Serdang, 43400 Serdang, Selangor, Malaysia.*
e-Mail: melati@upm.edu.my

SH-SY5Y is classified as a dopaminergic neuron model (Xie, at. al. 2010) (8) as they express the rate-limiting catecholaminergic enzyme tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) (Oyarce & Fleming, 1991) (9) and the dopamine transporter (DAT) that is only found in dopaminergic neurons of the central nervous system (Takahashi, et. al., 1994) (10). However, the cells are considered immature as they do not actively synthesize dopamine. Melino and co-workers (1997) (11) succeeded in differentiating the cells to a dopaminergic phenotype using all-trans-retinoic acid (ATRA). Earlier, Pahlman et. al. (1984) (12) found that ATRA conferred them with cholinergic neuronal phenotypes. The ability to synthesize measurable quantities of dopamine is the first step towards constructing a cell model of AADC deficiency. This study used L-DOPA to induce measurable synthesis of dopamine, which was then alumina extracted and identified using HPLC-ECD. To the best of our knowledge, no work has been done previously that addresses the dopaminergic synthesis potential of L-DOPA on SH-SY5Y cell cultures.

We also looked for the expression of markers of mature neurons in the cells. These are frequently employed to characterize the differentiation of previously immature cells. Four genes were chosen: growth-associated protein (GAP-43), microtubule-associated protein tau (MAPT), synaptophysin (SYP) and RNA binding protein, fox-1 homolog 3 (RBFOX3) based on previous works (Xie et. al., 2010 (8) and Constantinescu et. al., 2007 (13)). RT-PCR is the detection method of choice to highlight functionally expressed proteins in an organism.

2.0 Materials and methods

2.1 Cell Culture

SH-SY5Y (ATCC, USA) was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with Ham's F-12 medium and 10% fetal bovine serum (FBS) (PAA Laboratories GmbH, Austria) and 1% penicillin/streptomycin 100X (PAA Laboratories GmbH, Austria). Media was replaced every 5 days. Cells were seeded in TC25 culture flasks at 1 X 10^5 density and grown in 5% CO_2 and 95% air at 37° C. After propagation, cells were transferred to 6-well plates (Lifesciences, South Korea) for the purposes of these experiments. Subculturing was done when cells reach 80% confluence.

2.2 Western Blot

The aim of this experiment was to determine the presence of the dopaminergic pathway proteins in SH-SY5Y cells to justify its use as a model system for this work. Positive detection is sufficient to qualify a protein for further study. Proteins chosen to indicate a functioning dopamine synthesizing machinery are AADC/dopa decarboxylase (DDC), DBH and TH. Immunoblotting followed established reported protocols (www.abcam.com). All antibodies were obtained from Abcam, UK. Protein concentrations were standardized and loaded 10µg per well. Samples were loaded in triplicate wells. Blots were incubated with antibodies against AADC/DDC (#ab3905; 1:1,000 in 5% non-fat milk powder in Tris-buffered saline TWEEN-20 - TBST), DBH (#ab27956; 1:1,000, in 5% non-fat milk powder in TBST), TH (#ab41528; 1 µg/ml, in 5% non-fat milk powder in TBST) respectively. Next, secondary labeling followed with HRP (#ab6728; 5,000:5,000 in 5% non-fat milk powder in TBST) and detection under fluorescence. Concurrently β-actin was probed for each protein.

2.3 L-DOPA and Benserazide

DL-DOPA (DL-β-3,4-dihydroxyphenyl alanine) (Sigma-Aldrich, USA) has the molecular weight 197.2. All tests were carried out on 80% confluent cells growing in 6-well plates. Cells were prepared in triplicates. For 1 mg/ml L-DOPA, dissolve 5 mg L-DOPA in 5 ml complete media. Dispense 1 ml L-DOPA/media mixture into each well. Wells were labeled T_0, $T_{0.5}$, T_1, T_2, T_4 and T_6 representing incubation times (h). For T_0, media present in the well at the start of the experiment was pipetted and transferred to a 15 ml centrifuge tube, foil-wrapped and kept at -80°C before alumina extraction.

Benserazide (DL-serine 2-(2,3,4-trihydroxybenzyl) hydrazide HCl) (Sigma-Aldrich, USA) 0.6 mg/ml was added together with L-DOPA 0.25 mg/ml into the complete cell media in a cocktail and incubated.

2.4 Alumina Extraction

This methodology was optimized for 5 ml sample volume and has been modified from the original paper (Anton & Sayre, 1962) (14). Cells were grown in 6-well culture plates with 1 ml complete media with or without additional test substance(s). After exposure to test substance(s), the media was removed and transferred to a plastic 15 ml screw capped centrifuge tube. These tubes were protected from light and stored at -80°C prior to alumina extraction.

Immediately before alumina extraction, thaw frozen media and an appropriate amount of internal control (10 µg/ml dihydroxybenzylamine, DHBA, Sigma-Aldrich, USA). To each sample tube, add 1M sodium phosphate buffer pH 5 (adjusted with NaOH), 5% EDTA, alumina (aluminum oxide 90 active neutral) (Merck KGaA, Darmstadt, Gemany) and make up to 5 ml with dH_2O. Next, add 10 µg/ml DHBA internal control. Finally, add 900 µl 5M NaOH and two drops 1% ethanolic phenolphthalein. Cap tubes and mix well. A light pink colour should develop indicating a pH change to 8.

Invert-shake all tubes vigorously for 2 minutes and centrifuge at 1,500 rpm for 2 minutes. Remove the supernatant and perform two rounds of water washes with 5 ml dH_2O. Shake to mix and centrifuge at 1,500 rpm for two minutes each time. Remove water without disturbing the alumina pellet. Add 0.2M HCl to elute catecholamines from the alumina. Vortex vigorously for two minutes. Then, centrifuge the slurry at 2,500 rpm for 2 minutes. Pipette the supernatant into Eppendorf spin tubes. Centrifuge tubes at maximum speed (12,000 rpm) for 2 minutes to pellet any carry-over alumina. Pipette the final supernatant into fresh Eppendorf tubes. Store the extracted catecholamines at -80°C before HPLC analysis.

2.5 HPLC-ECD

Sample separation was done on an XBridge Shield RP18 3.5 µm, 4.6 X 100 mm, (Waters, BEH Technology Ireland) column. 1ml/min flow rate was generated and maintained by an LC-10AP (Shimadzu Scientific Instruments, US) pump. Mobile phase 500 ml was prepared by mixing 15 ml acetonitrile, 10 ml sodium phosphate buffer, pH 3.2, 0.8 ml 10% SOS and 1 ml EDTA, 5% in ddH_2O and filtered through a 0.2 µm pore size Whatman (GE Healthcare, US) nitrocellulose filter disc. Noradrenaline elutes first, followed by adrenaline, DHBA and dopamine. Samples are oxidized with an optimized applied potential of 0.6V by the LC1260 electrochemical detector (GBC Scientific

Equipment, Australia) and a silver, silver chloride (Ag/Ag^+) reference electrode. Sample detection is on an integrator in the form of peaks (C-R6A Chromatopac, Shimadzu Scientific Instruments, US).

2.6 Markers of Mature Neurons

RNA harvesting and extraction from neuroblastoma cells were done according to the protocol of RNEasy Plus Mini Kit (QIAGEN, Germany). Primers were designed specifically for this study using free online programs. Sense/forward primer sequence for GAP-43 is AAG AAG GAT GAA GCC CCT GT. Reverse or antisense sequence is CTG CTT CGG CAG TAG TGG T. Sense primer sequence for MAPT is AAA TAG GCC TTG CCT TAG CC and its antisense primer has the sequence CTC CTT TGC TCC AGA ACT GC. SYP sense primer sequence is GGC TTT AAG CGA GGC AGA AT with the antisense sequence of GGC TCT GGT ACC AAT CCA AG. RBFOX3 sense primer sequence is ACC ACA CTT CAG GGA GAT GG and antisense primer CAG TGG CTG TCT GTC TTG GA. The wells were pre-heated to 50°C and maintained for the duration of sample loading. This activates reverse transcription for 30 minutes followed by PCR initiation at 95°C for 15 minutes. This was followed by 35 cycles at 94°C, 55°C and 72°C respectively. After the last cycle, a final 10 minutes extension at 72°C and cool down to 4°C. The annealing temperature for *GAP-43* and *SYP* were optimized at 55°C and 35 cycles, *MAPT* at 55°C and 37 cycles and *RBFOX3* at 60°C at 37 cycles.

3.0 Results

3.1 Western Blot

All three proteins probed for to determine the presence of a dopaminergic pathway were identified (Figure 1). AADC/DDC, DBH and TH proteins were identified in SH-SY5Y cells based on the presence of bands on western blot.

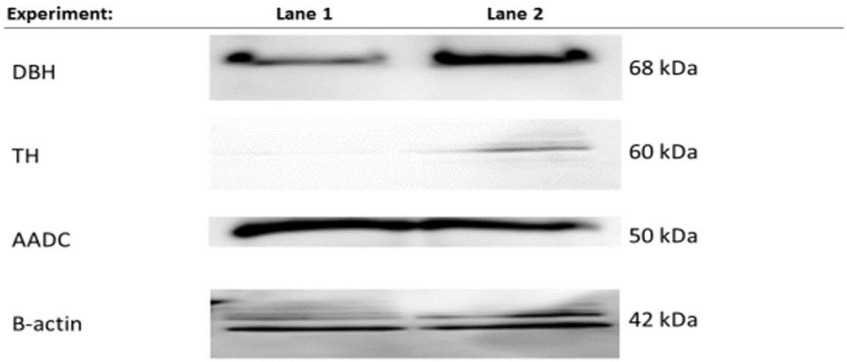

Figure 1: Western Blot of dopaminergic proteins.
Each blot was loaded in duplicate lanes with the same protein extract.

3.2 Dopaminergic Differentiation of SH-SY5Y

No measurable amount of dopamine was detected in ATRA-treated cells which was extracted and alumina-concentrated for HPLC with ECD analysis (Fig. 3). However, ATRA treatment did induce cells to stop proliferating (Fig. 2A). Cell growth is assumed to have been arrested at G1 cell cycle phase as reported by Melino (1997) (11). Cells also appear to have increased in size with neurite elongation and increased branching. Compare Figure 2A with Figure 2B where cells grew normally without the addition of ATRA.

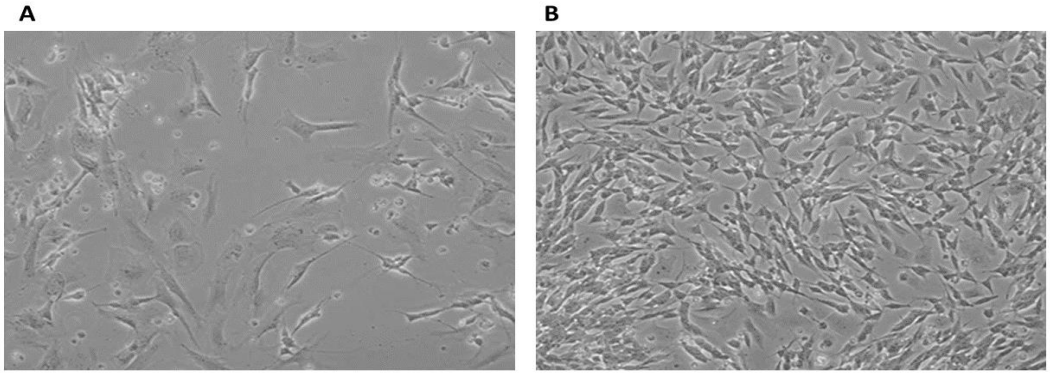

Figure 2: SH-SY5Y cell with and without *all-trans* retinoic acid treatment: At day-8 after ATRA treatment (A) and without ATRA treatment (B) at 10X magnification. Cells with ATRA are observed to be sparse in number, have larger cell bodies and longer neurites. Cells without ATRA have propagated in number.

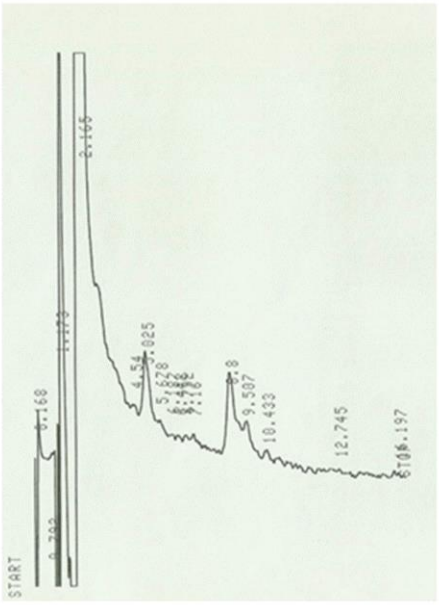

Figure 3: Chromatogram of ATRA-treated cells: HPLC-ECD of alumina-extracted media from cells treated with ATRA did not detect any dopamine, which would be expected to elute at 12 minutes.

3.3 Potentiation With L-DOPA

Dopamine production was detected as early as the second time point (Fig. 4A) at $T_{0.5}$ (30 minute incubation).

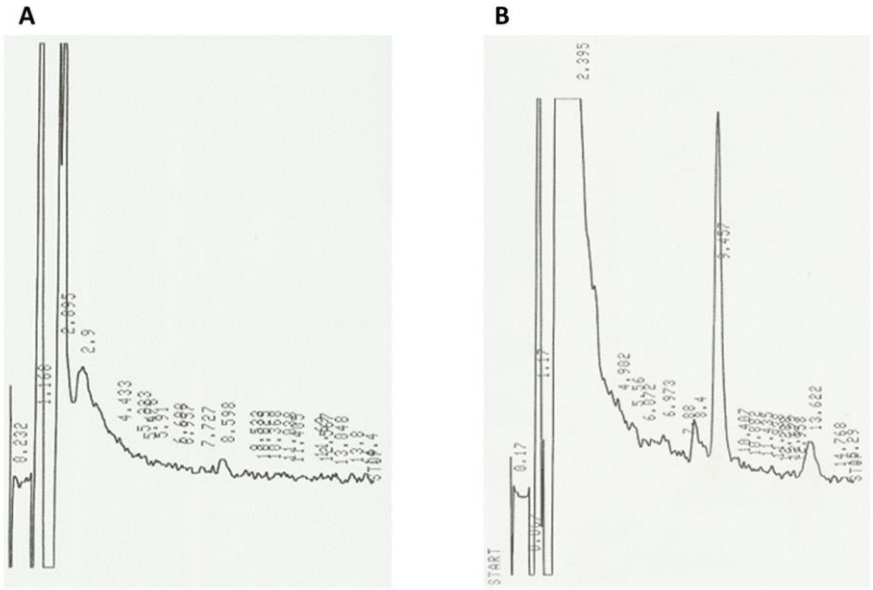

Figure 4: L-DOPA-treatment: (A) dopamine production in SH- SY5Y cells, after 30 minutes incubation eluted at 13.62 minutes. The tall spike at 9.45 minutes was the internal control DHBA. As a comparison, no dopamine was detected in cells not treated with L-DOPA (B).

3.4 Benserazide Inhibition

Benserazide inhibits dopamine production by inhibiting the action of AADC enzyme. This is observed by the downward logarithmic curve generated from collective data in Fig. 6. This graph was generated from various amounts of benserazide and measuring residual dopamine synthesis at each inhibitor concentration. Logarithmic inhibition curves will slope towards without ever reaching the x-axis. The value at which benserazide exert 50% inhibition against L-DOPA is 0.22 mg/ml, making it a very potent inhibitor (data not shown).

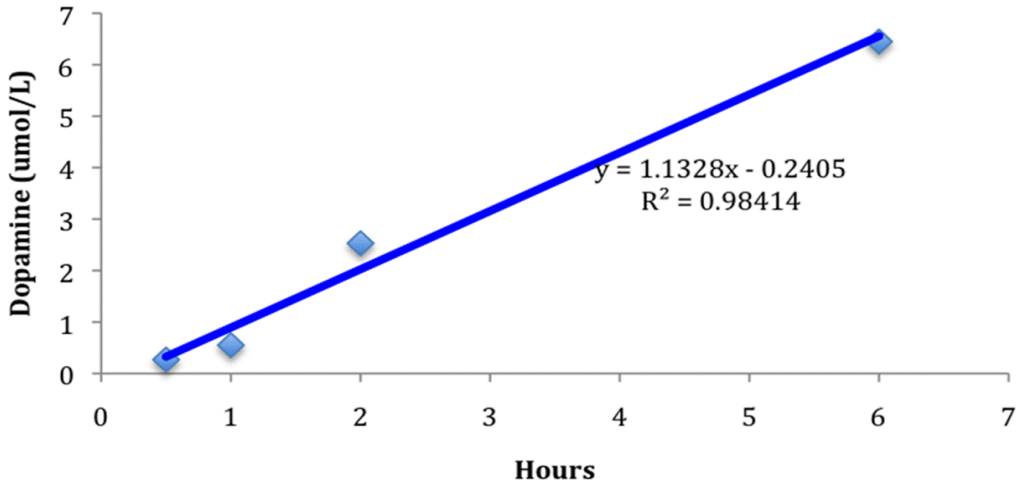

Figure 5: Time course for dopamine production with 1 mg/ml L-DOPA: concentration increased with longer incubation duration. Dopamine synthesis was linear up to the fifth time point T_4.

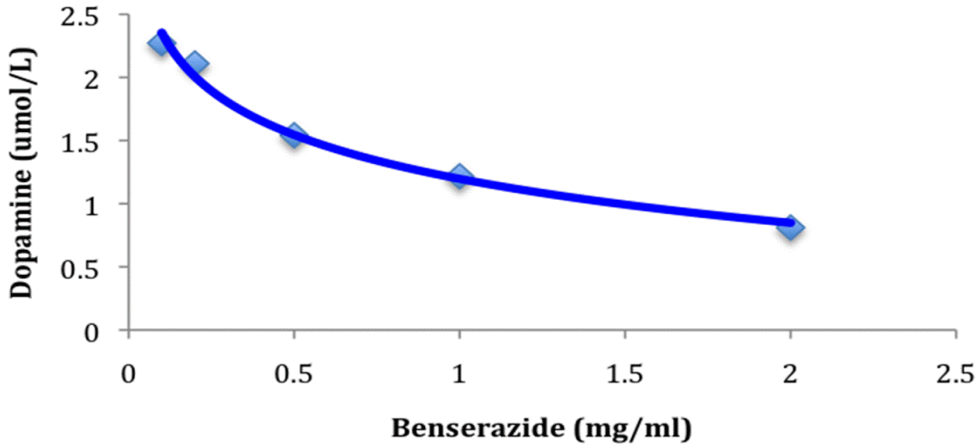

Figure 6: Benserazide inhibition of dopamine: benserazide at increasing concentrations reduce L-DOPA dopamine synthesis in SH-SY5Y.

3.5 Markers of Mature Neurons

RT-PCR experiments were designed to test for differences in the expression of marker genes between neuroblastoma cells in normal complete media, neuroblastoma cells in 0.25 mg/ml L-DOPA where dopamine synthesis is initiated and neuroblastoma cells in 0.25 mg/ml L-DOPA and benserazide representing our AADC cell model where the dopamine synthesis initiation is blocked. The results are shown in Figure 7.

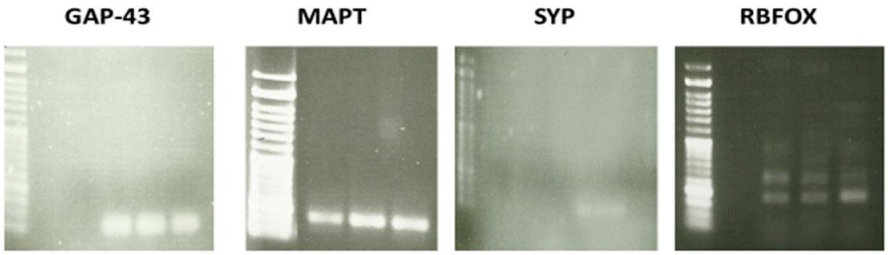

Figure 7: RT-PCR bands of markers of mature neurons: GAP-43 bands at 70 bp; MAPT bands at 154 bp; SYP bands at 63 bp; RBFOX with non-specific bands, one of it the target protein at 178 bp. All agarose gels were loaded with RT-PCR products in the order Lane 1: un-treated cells, Lane 2: cells treated with L-DOPA, Lane 3: cells treated with L-DOPA and benserazide.

4.0 Discussion

In this work, western blot was used to screen for the presence of dopaminergic pathway proteins of interest. As such, bands observed were not analyzed for protein concentration. A qualitative approach was sufficient. SH-SY5Y cells stayed true to their reputation as a dopaminergic cell line by expressing easily detectable amounts of the requisite catecholaminergic enzyme AADC also known as dopa decarboxylase. Dopamine β-hydroxylase (DBH) was also detected with ease and at relatively high amounts as evidenced by the thickness and

brightness of its protein band. DBH is the enzyme needed to convert dopamine to norepinephrine (NE) and subsequently to epinephrine (EPI). In the absence of DBH, neither NE nor EPI can be synthesized. Both AADC and DBH required relatively low amounts of antibody to probe for their respective proteins. It hints at the abundance of these proteins within the cells.

Tyrosine hydroxylase (TH) on the other hand was not as easy to detect. As the rate-limiting enzyme of the dopaminergic pathway, TH facilitates the conversion of the amino acid tyrosine to L-DOPA, the dopamine precursor. Frequently, TH-content is used as a marker for dopaminergic activity in cells. However, the presence of TH does not guarantee dopaminergic activity as demonstrated in various non-dopaminergic brain regions with TH expression. Conversely, yet other dopaminergic brain regions do not express TH activity (Bjorklund & Dunnet, 2007) (15). The low and non-specific expression of TH in SH-SY5Y may account for the difficulty in transforming the cells to a more dopaminergically mature cell state using all-trans-retinoic acid. Instead, when L-DOPA was used as a source of excess substrate, the cells readily synthesized large amounts of dopamine.

Cells in Figure 2A and 2B were of the same subculture and seeding density. They were grown in separate wells one in the presence of ATRA and the other without. After eight days of ATRA treatment, cells in Figure 2A appear to have increased in cell body size with neurite elongation and increased branching. This is consistent with reports from previous ATRA differentiation experiments (Melino et. al., 1997 (11); Maruyama et. al., 1997 (16); Nicolini et. al., 1998 (17), Constantinescu et. al., 2007 (13) and Korecka et. al. (2013) (22)). ATRA also caused the cells to stop proliferating. Cell growth is assumed to have been arrested at the G1 cell cycle phase as reported by Melino (1997) (11), Cheung (2009) (23) and Qiao (2012) (24). This can be observed in the relatively lower cell density in Figure 2A compared to Figure 2B. Without exposure to ATRA, these cells appear to have continued propagating without gains in cell body size or neurite length. ATRA precipitated a morphological change in our cells without the desired dopamine production. All-trans retinoic acid was deemed unsuccessful as a dopaminergic differentiation agent for our SH-SY5Y cells as evidenced by the lack of detectable dopamine.

SH-SY5Y cells were successfully made to synthesize dopamine with the administration of L-DOPA into its growth media. 1 μg/ml L-DOPA effectively precipitated dopamine production to levels detectable by HPLC with ECD in as little as 30 minutes. Consistent with available data, dopamine started to breakdown after 4 hours of exposure to oxygen. Oxidized dopamine produces dopachrome, a brownish-black substance whose dark hue correlates with the degree of oxygenation (Byszewska & Kanska, 2013) (25). In this study we refrained from adding anti-oxidants to the media to prevent dopamine degradation. A side study we conducted identified ascorbic acid as being the most effective at prolonging dopamine stability (Khalid & Earl, 2012) (18). This however, is feared to adversely affect the pH levels of the media and hence, cell integrity. Therefore, time point experiments were designed to run no longer than 6 hours. L-DOPA treatment of SH-SY5Y forms the basis of our AADC cell model whereby an available system readily produces dopamine with treatment.

The mere fact that L-DOPA treatment resulted in dopamine production by SH-SY5Y cells on itself is sufficient to indicate the experiment's success. Measurement of dopamine produced is not within the scope of the present study.

With the presence of L-DOPA in its growth media, SH-SY5Y synthesized measurable amounts of dopamine. Benserazide addition subsequently quells this effect, effectively mimicking albeit imprecisely, the dopamine deficient state of AADC deficiency. The combined effects of L-DOPA and benserazide on SH-SY5Y cells thus mimic the blocked dopamine state of AADC deficiency. Therein, our cell culture model of this disease was devised.

Based on RT-PCR results, our SH-SY5Y neuroblastoma cells express four markers of mature neuronal cells even before being subjected to any differentiation regimes. This include the growth-associated protein (GAP-43) (Benowitz and Routtenberg, 1997) (19), microtubule-associated protein (MAPT) (Xie et. al., 2010) (8), synaptophysin vesicle protein (SYP) (Cassiman et. al., 1999) (20) and recombinant FOX-3 (RBFOX3) whose product shares near perfect protein homology with neuronal nuclei (NeuN) (Kim et. al., 2009) (21). This finding contradict others that could only find these markers once cells have undergone ATRA differentiation.

Previous studies using SH-SY5Y as a dopaminergic model system have always considered the cells at native state to be immature (Xie et. al., 2010 (8) and Constantinescu et. al., 2007 (13)). This is because despite being dopaminergic, the cells do not possess all the characteristics of adult neurons of the brain. Of particular interest to the current study, SH-SY5Y does not produce dopamine even when Western blotting results show the presence of TH, DBH and AADC/DDC which constitute the dopamine synthesis mechanism. RT-PCR showed equal expression of our chosen marker genes of mature dopaminergic neurons in untreated SH-SY5Y cells and those treated with L-DOPA and L-DOPA with benserazide. It is interesting to note that multiple groups only detect these markers in mature, differentiated SH-SY5Y cells (Pahlman et. al., 1984 (12), Gimenez-Cassina et. al., 2006 (26); Cheung et. al., 2009, Xie et. al., 2010 (27) and Shipley et. al., 2016) (28)) while they were readily present in our cells with no modification. L-DOPA treatment does not induce the cells to gain a more neuronal morphology with longer neurites. It also does not halt cell proliferation as treatment with retinoic acid does. Treatment with L-DOPA however does enable the cells to synthesize detectable levels of dopamine. Subsequent treatment with the AADC enzyme inhibitor benserazide abolishes this dopamine production effect. As such, our cellular model for AADC deficiency was devised. It can be concluded that dopamine production in SH-SY5Y cells is not solely dependent on 'conventional' differentiation using currently available agents. Dopamine production may also not be the sole purview of 'mature' dopaminergic cells as our cells were obviously undifferentiated. When the dopamine substrate L-DOPA was added in excess of physiological levels, the existing dopaminergic pathway must have become initiated to begin decarboxylating it into detectable amounts of dopamine. Also, the current dogma of RNA transcription definitively leading to protein translation may no longer hold true.

5.0 Conclusion

SH-SY5Y cells were made to synthesize dopamine at levels detectable by HPLC-ECD which is then quelled using benserazide to mimic the dopamine-deficient state of AADC deficiency. Dopamine synthesis was not due to cellular differentiation but rather the addition of excess substrate. Together, L-DOPA treatment followed by benserazide inhibition formed our cell model of AADC deficiency.

A cell model may provide a testing avenue to assess medications used to treat AADC deficiency and to screen potential new drugs. Currently, therapeutic strategies rely on Parkinson's treatments which lack safety trials and recommended dosages for pediatric use. Anti-Parkinson's drugs also do not address the inability to decarboxylate L-DOPA in AADC deficient individuals. Together, they account for the dismal treatment outcome and substantial side-effects from its use in AADC deficient children. Before agents become available that specifically address and ideally treat this condition, a cell model may be useful to screen medications for their capacity to increase dopamine level.

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7.0 Disclosures

The authors declare no conflict of interest.

8.0 References

Swoboda, K. J., Saul, P., McKenna, K. & Speller, N. B. Aromatic L-amino acid decarboxylase deficiency: overview of clinical features and outcomes. *Annals of Neurology*. 2003; **54(6):** S49-S55.

Lin, M. T. Effects of brain monoamine depletions on thermoregulation in rabbits. *American Journal of Physiology*. 1980; **238:** R364 – R371.

Pons, R., Ford, B., Chiriboga, C. A., Clayton, P. T., Hinton, V., Hyland, K., Sharma, R. & De Vivo, D. C. Aromatic L-amino acid decarboxylase deficiency. Clinical features, treatment and prognosis. *Neurology.* 2004; **62:** 1058 – 1065.

Allen, G. F. G., Land, J. M. & Heales, S. J. R. A new perspective on the treatment of aromatic L-amino acid decarboxylase deficiency. *Molecular Genetics and Metabolism.* 2009; **97:** 6 – 14.

Voong, L. & Eriksson, T. Is rat brain content of large neutral aminoacids (LNAAs) a reflection of plasma LNAA concentrations? *Journal of Neural Transmission of Genetic Selection.* 1992; **87:** 133 – 143.

Korenke, G. C., Christen, H. J., Hyland, K., Hunneman, D. H. & Hanefeld, F. Aromatic L-amino acid decarboxylase deficiency: an extrapyramidal movement disorder with oculogyric crises. *European Journal of Pediatric Neurology.* 1997; **1:** 67 – 71.

Saxena, P. R. Serotonin receptors: subtypes, functional responses and therapeutic relevance. *Pharmacological Therapeutics*. 1995; **66:** 339 – 369.

Xie, H.-R., Hu, L.-S. and Li, G.-Y. SH-SY5Y human neuroblastoma cell line: *in vitro* cell model of dopaminergic neurons in Parkinson's disease. *Chinese Medical Journal*. 2010; **123 (8):** 1086 – 1092.

Oyarce, A. M. and Fleming, P. J. Multiple forms of human dopamine beta-hydroxylase in SH-SY5Y neuroblastoma cells. *Archives of Biochemistry and Biophysiology.* 1991; **290:** 503 – 510.

Takahashi, T., Deng. Y., Maruyama, W., Dostert, P., Kawai, M. and Naowi, M. Uptake of a neurotoxin candidate – (R)-1,2-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline into human dopaminergic neuroblastoma cells by dopamine transport system. *Journal of Neural Transmission. General Section.* 1994; **98:** 107 – 118.

Melino, G. Thiele, C. J., Knight, R. A. and Piacentini, M. Retinoids and the control of growth / death decisions in human neuroblastoma cell lines. *Journal of Neurooncology*. 1997; **31:** 65 – 83.

Pahlman, S., Ruusala, A. I., Abrahamsson, L., Mattson, M. E. and Esscher, T. Retinoic acid-induced differentiation of cultured human neuroblastoma cells: a comparison with phorbolester-induced differentiation. *Cell Differentiation.* 1984; **14:** 135 – 144.

Constantinescu, R., Constantinescu, A. T., Reichmann, H. and Janetsky, B. Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. *Journal of Neural Transmission*. 2007; Supplementary 72: 17 -28.

Anton, A. H. and Sayre, D. F. A study of the factors affecting the aluminum oxide trihydroxyindole procedure for the analysis of catecholamines. *Journal of Pharmacology and Experimental Therapeutics.* 1962; **138:** 360 – 374.

Bjorklund, A. and Dunnett, S. B. Dopamine systems in the brain: an update. *Trends in Neurosciences*. 2007; **30(5):** 194 - 202.

Maruyama, W., Benedetti, M. S., Takahashi, T. and Naoi, M. A neurotoxin N-methyl(R)salsolinol induces apoptotic cell death in differentiated human dopaminergic neuroblastoma SH-SY5Y cells. Neuroscience Letters. 1997; **232:** 147 – 150.

Nicolini, G., Miloso, M., Zoia, C., Di Silvestro, A., Cavaletti, G. and Tredici, G. Retinoic acid differentiated SH-SY5Y human neuroblastoma cells: an in vitro model to assess drug neurotoxicity. *Anticancer Research*. 1998; **18:** 2477 – 2481.

Khalid, M. and Earl, J.W. Dopamine preservatives in cell culture. Proceeding from AACB '11: *The Australasian Association of Clinical Biochemists*. 2011; Sydney, Australia.

Benowitz, L. I. and Routtenberg, A. GAP-43: an intrinsic determinant of neuronal development and plasticity. *Trends in Neuroscience*. 1997; **20:** 84 – 91.

Cassiman, D., van Pelt, J., De Vos, R., Lommel, F. V., Desmet, V., Yap, S-H. and Roskams, T. Synaptophysin: a novel marker for human and rat hepatic stellate cells. *American Journal of Pathology*. 1999; **155(6):** 1831 – 1839.

Kim, K. K., Adelstein, R. S. and Kawamoto, S. Identification of neuronal nuclei (NeuN) as Fox-3, a new member of the Fox-1 gene family of splicing factors. *The Journal of Biological Chemistry*. 2009; **284(45):** 31051 – 31061.

Korecka, J. A., van Kesteren, R. E., Blass, E., Spitzer, S. O., Kamstra, J. H., Smit, A. B., Swaab, D. F., Verhaagen, J. and Bossers, K. Phenotypic characterization of retinoic acid differentiated SH-SY5Y cells by transcriptional profiling. *PloS One.* 2013; **8(5)**, e63862.

Cheung, Y. T., Lau, W. K. W., Yu, M. S., Lai, C. S. W., Yeung, S. C., So, K. F. and Chang, C. C. Effects of all-*trans*-retinoic acid on human SH-SY5Y neuroblastoma as *in vitro* model inneurotoxicity research. *Neuro Toxicology*. 2009; **30**: 127 – 135.

Qiao, J., Paul. P., *et al*. PI3K/AKT and ERK regulate retinoic acid-induced neuroblastoma cellular differentiation. *Biochemical and Biophysical Research Communications*. 2012; **424(3):** 421-426.

Byszewska, W. and Kanska, M. Studies on enzymatic oxidation of 3',4'-dihydroxy-L-phenylalanine to dopachrome using kinetic isotope effect methods. *Journal of Radioanalytical And Nuclear Chemistry.* 2014; **299(3):** 1373 – 1378.

Gimenez-Cassina, A., Lim, F. & Diaz-Nido, J. Differentiation of a human neuroblastoma into neuron-like cells increase their susceptibility to transduction by herpesviral vectors. *Journal of Neuroscience Research*. 2006; **84(4):** 755-767.

Cheung, Y-T., Lau, W. K-W., *et. al*. Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *Neurotoxicology*. 2009; **30(1):** 127-135.

Shipley, M. M., Mangold, C. A. and Szpra, M. L. Differentiation of the human neuroblastoma cell line. *Journal of Visualized Experiments*. 2016; (108), e53193, doi: 10.3791/53193.