Ruairidh Barlow

**Does a knockout of NDUFA2 Lead to Parkinson’s disease?**

**Introduction**

Parkinson’s disease (PD) affects 0.3% of the human population and 1% of people over the age of 60 1, 2. PD is classified as an age related neurodegenerative disease, a type of disease characterized by a deterioration of the structure and function of neurons that can lead to neuron cell death. Symptoms include tremors, stiffness, and slow movement3. Studies of PD note that the death of dopaminergic neurons in the substantia nigra pars compacta is a key feature in the pathology of the disease. The substantia nigra is a group of subcortical nuclei that is located in the midbrain. Formed by dopaminergic neurons (neurons that contain the neurotransmitter dopamine), the substantia nigra plays a key role in motor control. However, despite this information, the complete pathology of PD is still unknown.

 In 1979, it was discovered that the toxin, 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced PD like symptoms. MPTP is a toxin that inhibits the activity of complex I (a protein complex) in the electron transport chain found in mitochondria4. This observation has alluded to a possible cause of death for neurons in the substantia nigra, as well as shifted the scope of PD related research to focus more on complex I dysfunction, and by extension, mitochondrial dysfunction. However, the hypothesis that mitochondrial dysfunction (more specifically a dysfunction in complex I) may play a role in the pathology of PD has yet to be confirmed as the root cause of PD.

 Complex I (Nicotinamide adenine dinucleotide (NADH):ubiquinone oxidoreductase) is the first enzyme in the electron transport chain. Specifically located in the inner membrane of mitochondria, its overall function is to catalyze the transfer of electrons from NADH to coenzyme Q105. This process moves four protons across the inner membrane which causes a membrane potential to build, a vital first step in ATP production. Complex I is made up of 44 protein subunits, 7 of which are synthesized by the mitochondrial genome5. An important subunit is NDUFA2 (Figure 1).

Figure 1: Structure of NDUFA2 Protein

 The protein NADH dehydrogenase [ubiquinone] 1 alpha sub complex subunit 2 (NDUFA2) is encoded by nuclear DNA (nDNA), a type of DNA that is found in the nucleus of eukaryotic organisms, and is located on the long q arm (Figure 2) of chromosome 5 at position 31.2 and it span 2,422 base pairs6. It has been observed that NDUFA2 interacts with other subunits of complex I as well as plays an important role in the assembly of complex I6. Heo et al (2012) experiment on another protein that affected complex I assembly. DJ-1 was the protein in question, DJ-1 is a PD related gene, which produces a protein that is known to maintain mitochondrial function7. However, it is not known in what way it affects the assembly of complex I.

Figure 2: Diagram of a chromosome, note location of q arm1

For this experiment, DJ-1 null mice were provided for this experiment. Heo et al did not actually perform the gene manipulation but used established substantia nigra cell lines that were provided by a Dr. Son (Ewha Womans University in Korea). The first part of the experiment was accessing the formation of complex I in a DJ-1 null cell. Effects of a DJ-1 deficiency on the translation subunits of complex I was observed by comparing the protein concentration between a wild type (SN4741) and the DJ-1 null cell this was done by using a Bradford assay (see figure S1 description). Next Heo et al tested if complex I was assembled correctly. This was done using a blue native-polyacrylamide gel electrophoresis (BN-PAGE). BN-PAGE differs from SDS-PAGE because it allows for protein separation while maintain protein to protein interactions. The blue coloring is due to dye molecules binding to proteins which then forms a protein-dye complex. The formation of the complex stabilizes the negatively charged anionic form of the dye producing the blue color17. The second part of the experiment focused on the affects that an incorrectly formed complex I would have on the rest of the mitochondria. This was done by measuring the production levels of ATP, oxygen consumption, and membrane potential. Levels of oxygen consumption was measured using a Seahorse Bioscience XF24 analyzer (this machine uses a culture plate comprised of chambers that are used to temporarily hold the cells of interest. The chamber also has sensors that can detect the amount of oxygen used in cell respiration) 18, mitochondrial membrane potential was measured with a rhodamine 123 dye (molecule with a positive charge, whose partitioning is sensitive to proton gradients. For this experiment the mitochondrial specific fluorescent variant of the dye will be used, can be purchase from SIGMA-ALDRICH), and ATP production measured by making the created ATP luminescent by introducing rLuciferase/Luciferin (an enzyme that produces bioluminescence/a light producing compound) reagent with control amounts ADP in a buffer solution. ATP production levels were then measured with an illuminometer. This experiment will use CRISPR/Cas9 (Clustered regularly-interspaced short palindromic repeats) a genome editing tool (Figure 3) to perform a knockout of NDUFA2 in a SH-SY5Y cell and then observe the effects of the knockout using methods similar to ones used in Heo et al. Does a knockout of NDUFA2 lead to reduced complex I activity causing changes consistent with PD?

Figure 3: Cas9 protein about to excise target sequence. Note how target sequence is adjacent to PAM sequence.

**Experiment**

The objective of this experiment is to determine how a knockout of NDUFA2 will affect the formation of complex I, mitochondrial oxygen consumption levels, mitochondrial membrane potential, and ATP synthesis in a SH-SY5Y human neuroblastoma cell that has been used extensively for studies of PD. The effects will be compared to both a control and PD diseased dopaminergic cell. I hypothesize that a NDUFA2 null cell will exhibit similar levels of oxygen consumption, membrane potential, and ATP synthesis found in a PD cell.

Core Materials Needed

Figure 4: SH-SY5Y cells. “Neurites extend, reminiscent of dendrites and/or axons.” Kovalevich et al (2013)

The SH-SY5Y cell line (Figure 4) for humans will be purchased from SIGMA- ALDRICH.com. NDUFA2 - human gene knockout kit via CRISPR will be purchased from OriGene.com. Both a control and a PD patient dopaminergic neuron will be used for this experiment. However, to be used for this experiment they would have to be live, and not from a post mortem specimen.  Stem cell lines for both PD patients and disease-free patients are available at the Coriel biorepository. The actual dopaminergic neurons would have to be grown from these stem cell lines, however this process is beyond the scope of this proposal.

gRNA Design

An important first step to using CRISPR/Cas9 is the creation of a guiding RNA (gRNA). gRNA functions as a way to help target the specific gene that is to be mutated. For this experiment NDUFA2 is the gene that will be targeted. Using the ATUM gRNA Design Tool web tool8, optimum target sites for CRISPR/Cas9 were selected. The resulting sequence for targeting NDUFA2 is “CCAGAGCTTGGGCTGCACAT”, (Sequence relative to the PAM sequence shown in red: CCAGAGCTTGGGCTGCACATCGG9) located on Chromosome 5 at position 140,026,868 with a score of 100 (a higher score indicates that the gRNAs are less likely to exhibit off target activity) 9. The generated sequence follows the convention oligos, short sequences of DNA, are about 19 – 25 nucleotides long9. A key element to have in mind when creating gRNA, is that the target sequence is close to a 5’-NGG-3’ proto-spacer motif (PAM) sequence (Figure 3). PAM helps Cas9 proteins recognize the desired area in the genome. Without it Cas9 proteins cannot edit the genome9. Furthermore, it is essential that designed gRNA does not have sequence similarity to other genes. These two factors, help prevent off targeting effects, basically the mutating an undesired gene9. After the completion of oligo design, the oligo will be cloned into a CRISPR/Cas9 vector.

Insertion of Oligo (gRNA) into a CRISPR/Cas9 Vector

The cloning of gRNA into the CRISPR/Cas9 vector is done using plasmids. Plasmid are small DNA molecules within a cell that are separated from a chromosomal DNA and can replicate independently. Pertaining to CRISPR/Cas9, E.coli plasmids are cleaved with restriction enzymes and the prepared oligos are then combined together. The combing process is done by heating the DNA strands and then cooling them, this results in the accurate joining of oligos. The now double stranded DNA oligo are then cloned, the covalent linking of two ends of DNA, into the E. coli plasmids using a ligation buffer according to protocol9. This part of the procedure produces recombinant DNA that is then transferred into E. coli via electroporation (Figure 5). This technique uses electric fields which results in the increased permeability of cell membranes. In this case, small holes will be created in the cell wall of E. coli which will allow the plasmids to enter the cell.

Figure 5: Overview of electroporation

Infection of SH-SY5Y Cell Line

 SH-SY5Y cell lines will be cultured in a (DMEM/F-12 50/50) growth medium10. To deplete NDUDA2 expression with CRISPR/Cas9 successful recombinants (the Cas9 gRNA vectors) will be packaged into a lentivirus9. This virus will then infect the host’s (SH-SY5Y) genome, excising the target sequence. This will cause a break in the double stranded sequence that will be repaired by the cell. This repair will ligate the strands back together, this ligation usually results in small insertion/ deletion mutations that disrupt gene expression, causing the knockout9.

**The rest of the procedure will be performed for each cell type (control, PD, and experimental).**

Isolation of mitochondria

Cells suspension will be generated by using a cell scraper and will then be centrifuged (Figure 6) in buffer 1 (250 mM sucrose, 2 mM HEPES, pH 7.4, and 0.1 mM EGTA) 7. Pellets formed from this process will then were then homogenized, the process of making a mixture out of two non-soluble substances the same throughout, in the same buffer. Resulting homogenate will then centrifuged, the resulting supernatant will then be centrifuged a second time, resulting in a crude mitochondrial pellet. This pellet will then be suspended in buffer 2 (25 mM potassium phosphate, pH 7.2 and 5 mM MgCl2), and centrifuged one final time7. This pellet will be used in the analysis of complex I assembly.

Figure 6: Overview of centrifugation

Complex I Assembly and Protein Subunit Detection

This section of the procedure will focus on first observing if complex I was able to properly form and what subunits are present, testing if the knockout was successful or not. Both a one dimensional and a two dimensional BN- PAGE will be performed. The purpose of the one dimensional part is the isolation of the mitochondrial superstructures. The purpose of the two dimensional part is observing if the knockout was a success (Figure 7). The mitochondrial pellet will be made soluble by using sodium dodecyl maltoside (a type of detergent used for the solubilization and purification of membrane proteins). For the detection of mitochondrial supercomplexes, such as complex I, digitonin (a non-ionic detergent), has been observed maintain the individual stability of the complex’s subunits11, will be used in the lysis buffer (used to break open cells)7. The samples will then be centrifuged and the resulting supernatants will then be separated under native conditions in a first-dimension BN-PAGE. Coomassie blue dye (negatively charged, binds nonspecifically to all the proteins and most importantly does not act like a detergent. This maintains the individual complex structures during electrophoresis) is used in BN-PAGE. Therefore, the electrophoretic mobility and separation of the samples is determined by the negative charge of the bound Coomassie blue and the size and shape of each complex12.

Figure 7: How to read a Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE)

For 2D BN/SDS-PAGE, the part of the gel containing the protein complex of interest will be cut out form the BN-PAGE gel. That slice of gel is then placed in sodium dodecyl sulfate (SDS) buffer, which denatures the proteins, and then will be in incubated on a shaker. The gel will then be loaded into a well where the separation of the protein subunits will be facilitated by an extend duration of electrical flow (Figure 7). SDS makes the protein subunits uniformly negatively charged, and so the distance each subunit travels is determined by its size13.

The separated subunits will be desalted, to improve ionization efficiency, and moved onto ready-made matrix-assisted laser desorption/ionization (MALDI) target plates. Mass spectra will be acquired using a 4700 proteomic analyzer (Applied Biosystems, Framingham, MA, USA). Protein will then be identified using Mascot version 2.2 software (Matrix Science Inc., Boston, MA) 7.

Mitochondrial Oxygen Consumption

Mitochondrial oxygen consumption levels will be measured using a Seahorse XF24 Extracellular Flux Analyzer (Seahorse Bioscience Inc., USA) in 24-well plates at 37°C. All cell types were seeded in wells 18 hours prior to examination and grown until confluent. Before each measurement, the cells were washed and 590 µl of non-buffered medium was added to each well7. The XF24 analyzer (Figure 8) is able to measure changes in oxygen consumption by the cells that does not require any dyes or labels14.

Figure 8: XF24 Extracellular Flux Analyzer

Mitochondrial Membrane Potential

Cells will be grown in 6 well plates for 24 hours and washed with phosphate buffer saline (PBS) 3 times. Cells will then be stained with rhodamine 123 dye in an incubator for 15 minutes at 37°. Cells will then be dissociated using trypsining, centrifuged at 800× g at room temperature (RT), and resuspended in PBS. Samples will then be analyzed on a FACScan (Figure 9), used to measure dye accumulation on membrane15 (BD Biosciences, Bedford, MA, USA) and data analysis will be performed with BD FACSDiva software (BD Biosciences, Bedford, MA, USA)7.

Figure 9: FACScan

Mitochondrial Rate of ATP synthesis

In order to measure ATP synthesis in each cell type, cells will have to be collected using trypsinization, and then centrifuged. The resulting pellet will then be washed with glucose and a serum free medium15. Cells will then incubated in a buffer to make their membranes permeable. The cell pellet will then be resuspended in buffer A. Next, diadenosine pentaphosphate, malate, pyruvate, and succinate will be added to the cell solution7. ADP and another buffer containing luciferin and luciferase will then be added to the cell solution7. The addition of luciferin and luciferase is key to making the measured ATP luminescent. Immediately after this addition, levels of ATP production will be measured by an illuminometer.

**Discussion**

The hypothesis, a CRISPR/Cas9 NDUFA2 knockout in SH-SY5Y cells will exhibit reduced oxygen consumption, membrane potential, and ATP synthesis. To ensure the knock out is successful, a 2D BN-PAGE analysis will be performed to analyze the individual proteins expressed in the SH-SY5Y cell line. The second portion of the experiment is done is understand the results of this knock out. (1) Complex I formation; (2) Mitochondrial O2 consumption; (3) Mitochondrial membrane potential; (4) Mitochondrial ATP synthesis levels. This part of the procedure will be performed on the experimental SH-SY5Y cell line, the control patient dopaminergic neurons, and PD patient dopaminergic neurons. Results will be compared. Expected results would be 1) Decreased formation of complex I; (2) Lowered levels of mitochondrial O2 consumption; (3) Lowered levels of mitochondrial membrane potential; (4) Lowered levels of mitochondrial ATP synthesis levels.

The first potential problem of this experiment is the cost of all the components, which are rather expensive. Actual problems that this experiment might face would be killing the cell with a knockout of NDUFA2. NDUFA2 could prove to be such a vital subunit of complex I that not only a dysfunction might occur, complex I might not even work at all! This would be chalked up to experimenter error since the experiment carried out in Martins- Branco et al (2012) showed that a knockout of NDUFA2 did not kill the cell19. However, steps have been taken to create two “back up experiments” should this cell death still occur (knockout NDUFS6 or NDUFA8). Another possible experimental pitfall would lie in the BN-PAGE. Both a one and two dimensional BN-PAGE are long and tedious methods so should both be done carefully and with care to ensure expected results. A possible unexpected result would be the knockout of NDUFA2 having no impact on both complex I and experimental levels (1 – 4).

Despite the potential pitfalls of this experiment the relation between complex I and Parkinson’s disease need to be studied further. Further experimentation needs to be performed in order to determine whether Parkinsonism is just the expression of complex I dysfunction in dopaminergic neurons.

**References**

1. de Lau LM, Breteler MM. Epidemiology of Parkinson’s disease. Lancet Neurol 2006;5:525-535.
2. Tanner, C. M., & Aston, D. A. (2000). Epidemiology of Parkinsonʼs disease and akinetic syndromes. Current Opinion in Neurology, 13(4), 427-430. doi:10.1097/00019052-200008000-00010
3. Postuma RB, Berg D, Stern M, et al. MDS clinical diagnostic criteria for Parkinson’s disease. Mov Disord 2015;30:1591-1601.
4. Nicklas, W., Vyas, I., & Heikkila, R. E. (1985). Inhibition of NADH-linked oxidation in brain mitochondria by 1-methyl-4-phenyl-pyridine, a metabolite of the neurotoxin, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine. Life Sciences, 36(26), 2503-2508. doi:10.1016/0024-3205(85)90146-8
5. Wirth, C., Brandt, U., Hunte, C., & Zickermann, V. (2016). Structure and function of mitochondrial complex I. Biochimica et Biophysica Acta (BBA) - Bioenergetics, 1857(7), 902-914. doi:10.1016/j.bbabio.2016.02.013
6. NDUFA2 NADH:ubiquinone oxidoreductase subunit A2 [Homo sapiens (human)] - Gene - NCBI. (n.d.). Retrieved April 15, 2017, from https://www.ncbi.nlm.nih.gov/gene?Db=gene&Cmd=ShowDetailView&TermToSearch=4695
7. Heo, J. Y., Park, J. H., Kim, S. J., Seo, K. S., Han, J. S., Lee, S. H., . . . Kweon, G. R. (2012). DJ-1 Null Dopaminergic Neuronal Cells Exhibit Defects in Mitochondrial Function and Structure: Involvement of Mitochondrial Complex I Assembly. PLoS ONE, 7(3). doi:10.1371/journal.pone.0032629
8. (n.d.). Retrieved April 15, 2017, from https://www.atum.bio/eCommerce/cas9/input
9. NDUFA2 - human gene knockout kit via CRISPR. (n.d.). Retrieved April 29, 2017, from http://www.origene.com/CRISPR-CAS9/KN202715/NDUFA2.knockout
10. Bao, L., Chen, S., Conrad, K., Keefer, K., Abraham, T., Lee, J. P., . . . Miller, B. A. (2016). Depletion of the Human Ion Channel TRPM2 in Neuroblastoma Demonstrates Its Key Role in Cell Survival through Modulation of Mitochondrial Reactive Oxygen Species and Bioenergetics. Journal of Biological Chemistry, 291(47), 24449-24464. doi:10.1074/jbc.m116.747147Schagger, H. (2000). Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. The EMBO Journal, 19(8), 1777-1783. doi:10.1093/emboj/19.8.1777
11. Proteomics, C. (n.d.). 2D Blue Native. Retrieved April 29, 2017, from http://www.creative-proteomics.com/services/2d-blue-native-sds-page-for-complex-analysis.htm
12. Fiala, G. J., Schamel, W. W., Blumenthal, B. Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE) for Analysis of Multiprotein Complexes from Cellular Lysates. J. Vis. Exp. (48), e2164, doi:10.3791/2164 (2011).
13. University of Virginia School of Medicine. (n.d.). Retrieved April 29, 2017, from https://pharm.virginia.edu/facilities/seahorse-xf24-extracellular-flux-analyzer/
14. Vives‐Bauza, C., Yang, L., & Manfredi, G. (2007). Assay of Mitochondrial ATP Synthesis in Animal Cells and Tissues. Mitochondria, 2nd Edition Methods in Cell Biology, 155-171. doi:10.1016/s0091-679x(06)80007-5
15. Hodge, G. K., & Butcher, L. L. (1980). Pars compacta of the substantia nigra modulates motor activity but is not involved importantly in regulating food and water intake. Naunyn-Schmiedeberg's Archives of Pharmacology, 313(1), 51-67. doi:10.1007/bf005058
16. Chial, H. J.; Thompson, H. B.; Splittgerber, A. G. (1993). "A spectral study of the charge forms of Coomassie Blue G". Analytical Biochemistry. 209 (2): 258–266. doi:10.1006/abio.1993.1117
17. How the Seahorse XF Works SeahorseBioscience - <https://www.youtube.com/watch?v=rkMpnLL7LBw>
18. Martins-Branco, D., Esteves, A. R., Santos, D., Arduino, D. M., Swerdlow, R. H., Oliveira, C. R., . . . Cardoso, S. M. (2012). Ubiquitin proteasome system in Parkinson's disease: A keeper or a witness? Experimental Neurology, 238(2), 89-99. doi:10.1016/j.expneurol.2012.08.008
19. Figure 1: https://upload.wikimedia.org/wikipedia/commons/thumb/a/a5/Protein\_NDUFA2\_PDB\_1s3a.png/375px-Protein\_NDUFA2\_PDB\_1s3a.png
20. Figure 2: http://img.tfd.com/dorland/arm\_chromosome.jpg
21. Figure 3: https://www.abmgood.com/premadeICRISPR-new/images/CRISPR-Sorting-Feature-Knowledge-Base-Intro-CRISPR.png
22. Figure 4: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5127451/
23. Figure 5: http://www.btxonline.com/media/wysiwyg/faq\_page/electroporation-cartoon.png
24. Figure 6: https://adarshsomani02.files.wordpress.com/2015/02/centrifugation4.png
25. Figure 7: https://www.jove.com/video/2164/blue-native-polyacrylamide-gel-electrophoresis-bn-page-for-analysis
26. Figure 8: https://pharm.virginia.edu/files/2014/01/Seahorse.jpg
27. Figure 9: <https://upload.wikimedia.org/wikipedia/commons/thumb/1/13/FACS-toestel.JPG/300px-FACS-toestel.JPG05>