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The role of DJ-1 in enhancing mitochondrial quality control

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THE ROLE OF DJ-1 IN ENHANCING MITOCHONDRIAL QUALITY CONTROL

by

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DEDICATION

I dedicate this work to my Mother and Father

ACKNOWLEDGEMENTS

I could not have completed these experiments without the wonderful help and encouragement from my mentors Dr. Orian Shirihai Ph.D, Andrew Ferree, and Kyle Trudeau. Andrew and Kyle in particular taught me how to do this research from the most basic of techniques to the most advanced of data analysis. They are researchers and teachers of the highest caliber. All questions were welcomed and it was a pleasure to work with them. Sam Serada was also a terrific help due to his extreme enthusiasm and complete willingness to help me even though he was usually busy himself. Ashley, Amanda, Jibrin, Fernanda, Kiana, Nate, Noor, Rajween, Lindsay, Wei, Marc, and Eleni were also extremely helpful and made the lab environment very fun.

THE ROLE OF DJ-1 IN ENHANCING MITOCHONDRIAL QUALITY CONTROL

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ABSTRACT

DJ-1 is a cytosolic sensor for oxidative damage which acts on the Mitochondria. It works to curb the negative effects of high membrane potential in mitochondria, but the mechanism of action is still uncertain. This study measured DJ-1's potential in enchancing mitochondrial quality control in the context of pancreatic B-cells treated with a palmitate and glucose media to promote glucolipotoxicity (GLT). DJ-1 was proven capable of reversing GLT induced changed in mitochondrial morphology in the arenas of Feret's diameter, aspect ratio, and form factor. We also showed that the mitochondrial membrane potential did not vary with the presence or absence of DJ-1. In addition, DJ-1 was shown capable of limiting the upward boundary of GLT induced increase in mitochondrial membrane potential. Furthermore, an experiment using INS1 cells with GFP-LC3 showed that DJ-1 can decrease the average number of autophagosomes in the cell.

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ABBREVIATIONS

AP	Autophagosome
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CO2	Carbon Dioxide
dH2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
FFAs	Free fatty acids
GFP	Green Fluorescent Protein
GLT	Glucolipotoxicity
H2O2	Hydrogen peroxide
Hsp	Heat shock proteins
INS1	Insulinoma cell line 1
LB	Lysogeny broth
LC3	Microtubule-associated protein 1 light chain 3
LSM710	Laser scanning microscope 70
M	Molar
MFN1	Mitofusin 1

MFN2	Mitofusin 2
ML	Milliliter
Mm	Millimolar
mtDNA	mitochondrial DNA
MQC	Mitochondrial quality control
mV	Millivolt
Pink1	PTEN-induced kinase 1
PQQ	Pyrroloquinoline quinone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Rotations per minute
RPMI	Roswell Park Memorial Institute
TMRE	Tetramethylrhodamine, ethyl ester perchlorate
tRNA	Transfer RNA
US	United States
WHO	World Health Organization
WT	Wildtype

INTRODUCTION

Mitochondria

Mitochondria are organelles found in eukaryotic cells and possess two separate membranes. They contain their own DNA and also grow and reproduce independently from the rest of the cell. These qualities have lent support to the endosymbiotic theory that states that the mitochondria was once an oxygen producing, non-photosynthetic prokaryote which was engulfed by a eukaryotic cell, resulting in a symbiotic relationship (Embley 2006). Mitochondria perform many functions for the cell including fatty acid metabolism along with amino acid and iron-sulfur cluster production. Most notably, the mitochondria is responsible for producing the 'energy currency of the cell', ATP, through the process of oxidative phosphorylation. An unavoidable byproduct of this process is the generation of reactive oxygen species (ROS), which can disrupt the cell's activity and health greatly. Thus the mitochondria is both evolutionarily essential for eukaryotic cellular function and also a source of potentially damage causing molecules (Fischer et al 2012).

Quality Control

Mitochondrial quality control (MQC) is the set of pathways the cell employs in order to contain and reduce damage due to ROS at the molecular, organellar, and cellular level. While harmful in large amounts, low concentrations of ROS are necessary for several cell signaling pathways. Higher concentrations

can cause damage to lipids, proteins and also nucleic acids through the removal of electrons (Fischer et al 2012). In addition, there is a relationship between ROS concentrations and membrane potential ($\Delta\Psi_m$) in that depolarization of a mitochondria causes decreased ROS production (Joselin 2012).

One method the cell uses to enact MQC is by converting ROS into less harmful compounds. Superoxide is the major ROS created at the respiratory chain and can be converted into H2O2 with the help of the enzyme superoxide dismutase. The H2O2 can then then be acted upon in the mitochondria by the antioxidant enzymes peroxiredoxin or glutathione reductase in order to form water. Such scavenging methods are unable to fully quell the ongoing molecular damage brought on by ROS, but are able to slow down the process.

Another strategy of MQC is to repair damaged cellular components. In order to repair mitochondrial DNA (mtDNA), mitochondria utilize base excision repair, mismatch repair and direct reversal. Protecting mtDNA is essential to mitochondrial health since it codes for several mitochondrial tRNA, RNA, and proteins. In addition, chaperone proteins are able to rescue the three-dimensional structure of misfolded mitochondrial proteins. These heat shock proteins (Hsp) include Hsp22, Hsp60, and Hsp70. While some damage can be repaired, most proteins undergo irreversible damage from ROS and must be both removed and replaced (Fischer et al 2012).

Quality Control Pathway: Mitochondrial Dynamics

Another avenue of MQC involves the process of mitochondrial dynamics, which includes the fusion, fission, selective depolarization, and possible autophagy of mitochodnria (mitophagy). The act of fusion creates networks with continuous matrix lumen and membranes which allow for the rapid transfer of soluble proteins, and other solutes or metabolites. The exchange of mitochondrial DNA and membrane embedded proteins occurs much slower. This mutual exchange process lowers the heterogeneity of the healthy mitochondrial population. Fission is highly important because it produces unequal daughter mitochondria: each of the two groups has a $\Delta\Psi_m$ that usually differ by 5 mV or more. The group with the higher $\Delta\Psi_m$ is likely to undergo future fusion while the group with lower $\Delta\Psi_m$ is less likely to fuse. The mitochondria with decreased $\Delta\Psi_m$ hold a much greater amount of oxidized and damaged material and are much more likely to undergo autophagy. Thus, through numerous cycles of fusion and fission the quality and health of the mitochondrial population is further maintained (Twig et al 2008, Ferree et al 2012).

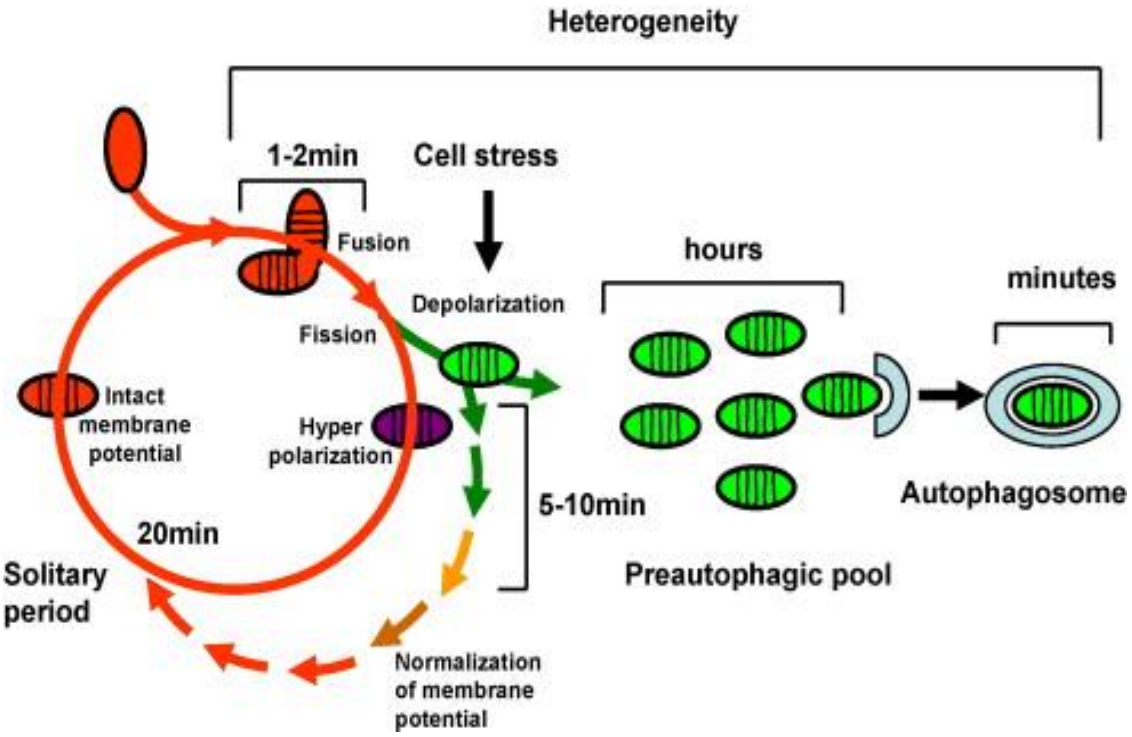


Figure 1: Mitochondrial life cycle. Figure taken from Wikstrom et al. 2009

Autophagy

Autophagy is the cellular method of recycling the components of damaged or unneeded organelles. The process involves the progression of the dual membrane phagophore to the autophagosome (AP). Upon acquisition of the damaged or unneeded cellular material, acidification occurs. Next, the AP fuses with the lysosome to produce the autolysosome, which degrades the aforementioned cellular components (Las et al 2011). In order to quantify the process of autophagy, the cytosolic protein marker for autophagy, LC3 (microtubule-associated protein 1 light chain 3), is used. Upon the onset of

autophagy, LC3 is lipidated and binds to the AP membrane, where it stays until it is either released or degraded.

DJ-1

DJ-1 is a cytosolic chaperone protein that acts as a sensor of mitochondrial oxidative stress and subsequently translocates to the mitochondria once it is oxidized (Dekker et al 2003). In the last few years, the function of DJ-1 has been more closely tied to the genes Pink1, and Parkin in the capacity of maintaining mitochondrial dynamics and MQC (Irrcher et al. 2010, Thomas et al. 2011). DJ-1, PINK1, and Parkin are three genes which cause parkinsonism when present in their recessive form. DJ-1 acts on mitochondria that have normal to higher membrane potential and higher ROS production, while PINK1 acts on mitochondria that have lower membrane potential and lower ROS levels. The downstream effector Parkin acts on the parallel DJ-1 and PINK1 pathways to maintain proper selective autophagic degradation of the mitochondria on both extremes (Ferree 2012). It is important to note that while Parkin can rescue the effect of both DJ-1 or Pink1, the opposite is not true since neither DJ-1 nor PINK1 can abrogate the effect of Parkin (Irrcher et al 2010; Dodson and Guo 2007). The loss of DJ-1 leads to disruptions in mitochondrial morphology and function such as loss of mitochondrial polarization, mitochondrial fragmentation, and lack of decreased autophagic clearance (Ferree 2012; Irrcher et al 2010; Thomas et al 2011). In addition, DJ-1 knockout in Drosophila has been

implicated in decreased AKT signaling (Yang et al 2005)(read in Dagda and Chu 2009). Furthermore, the antioxidant Pyrroloquinoline quinone (PQQ), was shown to increase the levels of the active reduced form of DJ-1 (Nunome et al 2008). Also, the amino acid cysteine at the site 106 is what allows DJ-1 to function in this capacity (Canet-Aviles et al 2004). Unfortunately, the precise pathway in which DJ-1 modulates ROS production is uncertain. Two potential sources of clarity in this regard involve modulation of uncoupling protein expression and also regulation of complex I activity (Hayashi et al 2009).

Diabetes

Diabetes is a significant public health concern affecting more than 347 million people worldwide, with the highest prevalence in India, China, and the United States(Danaei et al, WHO 2009). In 2004, and estimated 3.4 million people died worldwide from complications of diabetes (WHO fact sheet). According to the U.S. Centers for Disease Control and Prevention, diabetes affects 25.8 million people or 8.3% of the U.S. population, with 18.8 million diagnosed and 7 million undiagnosed (CDC). Furthermore, more than 79 million U.S. citizens are estimated to be pre-diabetic. Over time, diabetes can damage the heart, blood vessels, eyes, kidneys, and nerves (WHO 2009). Diabetes is the leading cause of kidney failure, non-traumatic lower limb amputations, and new cases of blindness among adults in the United States. In addition to the immediate health risks, the financial toll is a heavy burden. In 2012, the total cost

of diagnosed diabetes in the U.S. was estimated to be $245 billion with $176 billion in direct medical costs and $69 billion for reduced productivity.

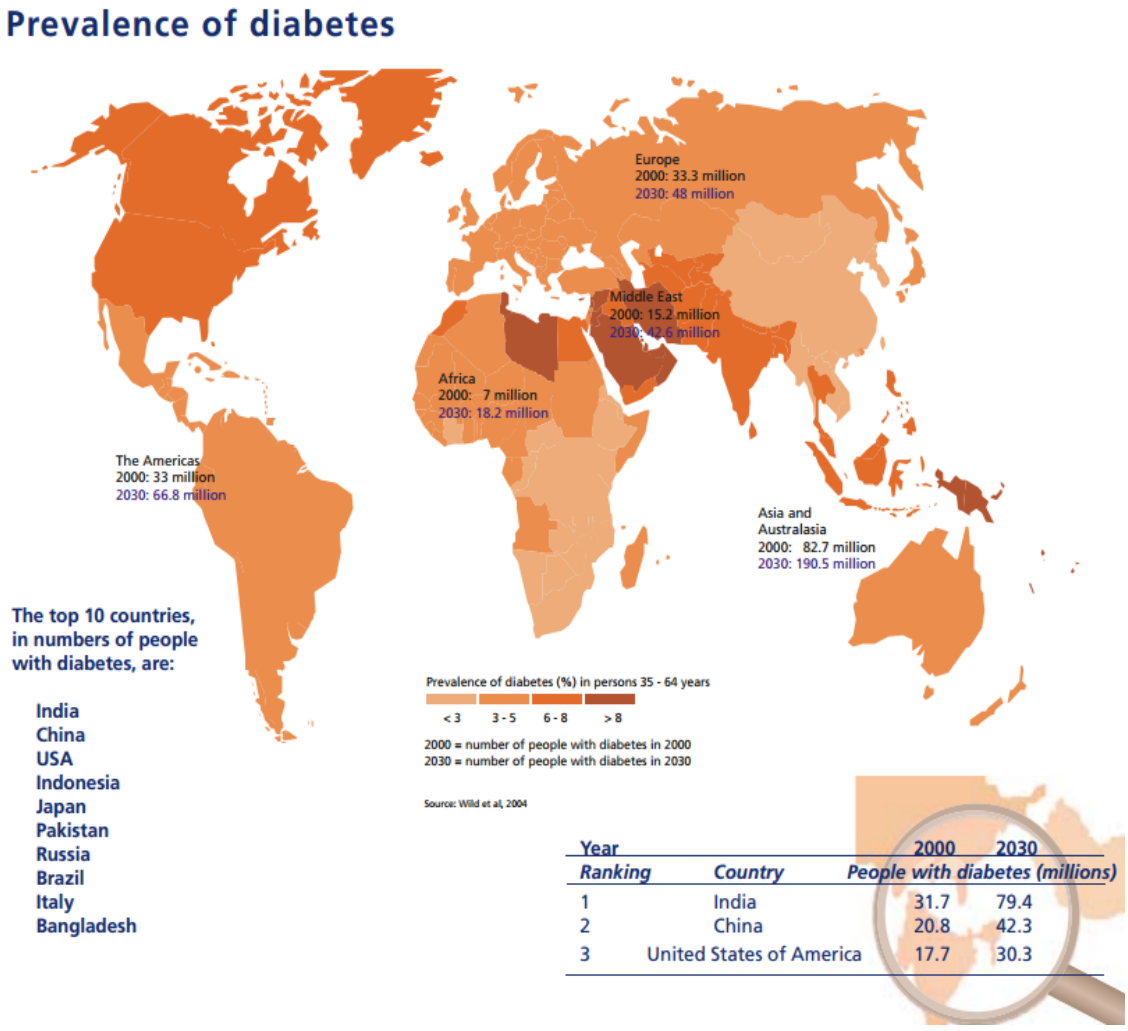


Figure 2: International prevalence of diabetes. Data includes adults age 35-64 in 2000 as well as a 2030 estimate. The darker areas represent areas of higher prevalence in terms of percentage of total individuals in the age group in

that country. Figure downloaded from " WHO at

http://www.who.int/diabetes/actionnow/en/DANbooklet.pdf

Modeling Diabetes

Type 2 diabetes involves a decrease in both pancreatic B-cell mass and function as well as an increase in peripheral insulin resistance. These factors result in a profound weakening of glucose homeostasis. These impairments in Type 2 Diabetes are often the result of a toxic overabundance of nutrients: free fatty acids (FFAs)("lipotoxicity), glucose ("glucotoxicity"), or both glucose and FFAs ("glucolipotoxicity"). The net result of these toxicities is an increased predilection for apoptotic cell death and reduced insulin secretion (Las et al 2011; El-Assaad et al 2003; Poitout et al 2008). Las et al showed that the fatty acid Palmitate could both increase the size and number of Aps due to increased content of AP substrates. They did this using INS1 treated with 0.4 mM palmitate for 14hours with GFP-LC3 as the method of tracking APs. In addition, palmitate was found to increase the rate of formation of APs without autophagy reaching a steady state in the first 24 hours (rate of formation = rate of degradation).

The Present Experiment

The point of this experiment is to gain a further I hypothesize that DJ-1 will be able to decrease the mean membrane potential of the mitochondrial population in cells with nutrient overload. Morphology was quantified in the INS1

cells lacking GFP-LC3 using three variables: Feret's diameter, aspect ratio, and form factor. Feret's diameter quantifies the longest line that can be drawn through a shape. Aspect ratio compares the length of the major axis to the minor axis such that a perfect circle has an aspect ratio of one. Form factor is equal to the theoretical area over the actual area or (Perimeter^2)/(4◦3.14◦ Area). I also hypothesize that DJ-1 will substantially reverse the fragmented morphological phenotype associated with nutrient overload and lead to more elongated structures. The other INS1 cell line used utilized GFP-LC3 in order to quantify the number of APs. I hypothesize that DJ-1 will attenuate the effects of nutrient overload on autophagy by increasing the size and number of the APs present in the cell.

Methods

DJ-1 DNA

The DNA used in these experiments were among nine samples of DJ-1 DNA constructs generously shared by the laboratory of Mark Cookson Ph.D at the National Institute of Health. Six of the constructs were pLenti 6 vectors (Invitrogen) for lentiviral based expression in target protein. The pLenti 6 vectors contained a C-terminus V5 epitope. The other three samples of DNA were pcDNA 3.2 vectors which are delivered to the cell via non-lentiviral transfection. These pcDNA vectors are advantageous in that they contain an ampicillin resistance gene and a pUC origin for selection and maintenance in E.coli. Out of these three vectors, the DJ-1 wild type gene and the DJ-1 C106A mutant were used. As these plasmids were spotted onto filter paper, each of the circled regions were cut out and placed into an Eppendorf tube. Then 50µl of QIAGEN elution buffer was added and the mixture was microcentrifuged for 1 minute at (rpm) in order to elute the DNA samples.

Transforming E.coli

Next, the DJ-1 WT and the DJ-1 C106A mutant were used to transform E.coli which was specifically grown for the growth and purification of plasmid DNA that will be digested by a restriction enzyme. This transformation method required adding 10ul of the DNA elution (or ligation?) product and 50ul of E.coli (gently thawed on ice since stored at -80°C) to an Eppendorf tube. After 30

minutes incubation on ice and 30seconds incubation in a 42°C water bath followed by another 2 minutes of incubation on ice the heat shock was complete. 200ul of S.O.C. medium was added and the solution was incubated in a shaking incubator (37°C, 225rpm) for 1 hour. Subsequently, 100ul of the E.coli/DNA solution was applied (dropwise with a sterile tip) to an agar plate which had been incubated at 37°C for the same hour and which contained ampicillin. The plate was evenly coated using a sterilized triangle scraper, the lid was sterilized in flame, and the plate was incubated at 37°C overnight.

Mini and Maxi Preps

In order to grow up a specific colony of recombinant bacteria, 5g of LB broth was added to 250ml of distilled water in a 1L Erlenmeyer flask. The flask was covered with both aluminum foil and autoclave tape and then autoclaved on a gravity 30 setting. After the flask cooled to room temperature, 250ul (1000x) of ampicillin was added and the mixture was inoculated by one colony from the aforementioned agar plate containing the E.coli/DNA. This LB media mixture was placed in a 37°C shaking incubator (300rpm) overnight. Next the bacterial pellet was harvested and the plasmid DNA was purified using a MiniPrep procedure (QIAGEN). The first steps of this procedure involved the re-suspension of the bacterial pellet in a buffer containing RNase A, the addition of an alkaline cell lysis solution followed by gentle mixing, and incubation at room temperature for 5 minutes. Next, a neutralization buffer was added to stop the cell lysis reaction,

the mixture was thoroughly mixed, and it was incubated on ice for 5 minutes. This mixture (1-2ml) was then microcentrifuged (12,000rpm) and the resulting clear supernatant was applied to an equilibrated QIAGEN column and allowed to enter the resin by gravity flow. Dyes, RNA, low molecular-weight impurities, and proteins were removed from the column using a medium-salt wash buffer and the DNA plasmid remaining on the resin of the column was eluted by a high-salt buffer. The DNA was desalted following addition of isopropanol and after microcentrifugation at 15,000x g rpm for 30 minutes and subsequent decanting, a DNA pellet was isolated. 70% ethanol was then added, the mixture was microcentrifuged at 15,000x g rpm for 10 minutes, and decanted and allowed to air dry for 5 minutes. The final DNA pellet was then dissolved in de-ionized water.

In order to verify that that bacterial colony used housed the correct DNA (as opposed to a source of contamination), the MiniPrep purified DNA was analyzed using gel electrophoresis. After verification of the bacterial colony, another round of LB media was cultured and inoculated from the same bacterial colony. The resulting bacterial pellet's DNA plasmid was purified using a MaxiPrep kit (QIAGEN), which used a larger quantity of the same reagents used in each step of the MiniPrep kit and thus required the use of a full sized centrifuge instead of a microcentrifuge. The purpose of the MaxiPrep was to increase the amount of transfection grade DJ-1 and DJ-1 C106A.

Transfection

The transfection reagent used in these experiments was Lipofectamine 2000 (Invitrogen). This reagent mediates the process of lipofection, where a cationic liposome complexes with anionic DNA in order for the DNA to overcome the electrostatic repulsion of the cell membrane and enter the cell. In addition, Lipofectamine 2000 is thought to help the DNA enter the nuclear envelope, allowing for highly efficient transfection. Furthermore, the transfection efficiency is influenced by factors such as cell density, DNA and liposome concentrations, DNA-liposome complexing time, and the inclusion or exclusion of media ingredients such as serum or antibiotics (Dalby 2004). Thus Opti-MEM reduced serum media (Invitrogen) was used to dilute Lipofectamine 2000. Approximately 700,000 cells, producing a 90-95% confluency, were plated into each of three wells in a 24 well plate for each of the two cell lines used in the experiment. DJ-1 plasmid DNA was cotransfected with mitochondrially targeted GFP for the INS1 cells or mitochondrially targeted DS Red for the LC3-GFP cells in order to determine transfection efficiency.

Cell lines

Cell lines used in these experiments were the pancreatic B-cell lines INS1 and also a line of INS1 cells with GFP-LC3. Both cell lines were bathed in an INS1 media consisting of RPM11640 medium (Invitrogen) supplemented by 10% FBS, 10mM HEPES buffer, 1mM pyruvate, 50uM 2-B-mercaptoethanol (added

working aliquot, not stock, in order to maintain effectiveness), 50 units/ml penicillin, and 50 ug/ml streptomycin. Cells were housed in 500ml cell culture flasks (Corning). When splitting flasks of cells approximately twice each week, trypsin was used to end the adherence of the cells to the flask. After about 5 minutes, cells were pipetted out, spun down, new media was added, and the resulting mixture was added to a fresh cell culture flask. Cells were stored in a incubator set at 37°C.

Palmitate and BSA

Palmitate (Sigma) was dissolved in DMSO to achieve a concentration of 0.4M and this solution was dissolved at 56°C in RPMI 1640 media containing 5% fatty acid-free BSA (Calbiochem) to make a 10x stock. RPMI 1640 media containing 5% BSA and 1% DMSO was used as a control. On the day of the experiment, the both stocks were added to a variation of the aforemention INS1 media containing only 1% FBS. In addition, 1M glucose was added to the palmitate containing media in a 1:100 ratio. These solutions were filtered and then added to their respective quadrant dish and remained there for approximately 18 hours before imaging.

TMRE

Tetramethylrhodamine, ethyl ester perchlorate (TMRE) is a cell permeant, cationic, red-orange dye that is taken up by active mitochondria in a membrane

dependent manner. A higher amount of TMRE present in mitochondria equates to a higher membrane potential. 1uL of the TMRE was added to 1000uL of DMSO and 10uL of the resulting solution was dissolved in 10mL of INS1 media, which was added to the cells 30 minutes prior to imaging. Since the TMRE is light sensitive, it was wrapped in aluminum foil during the preparation of the TMRE containing media.

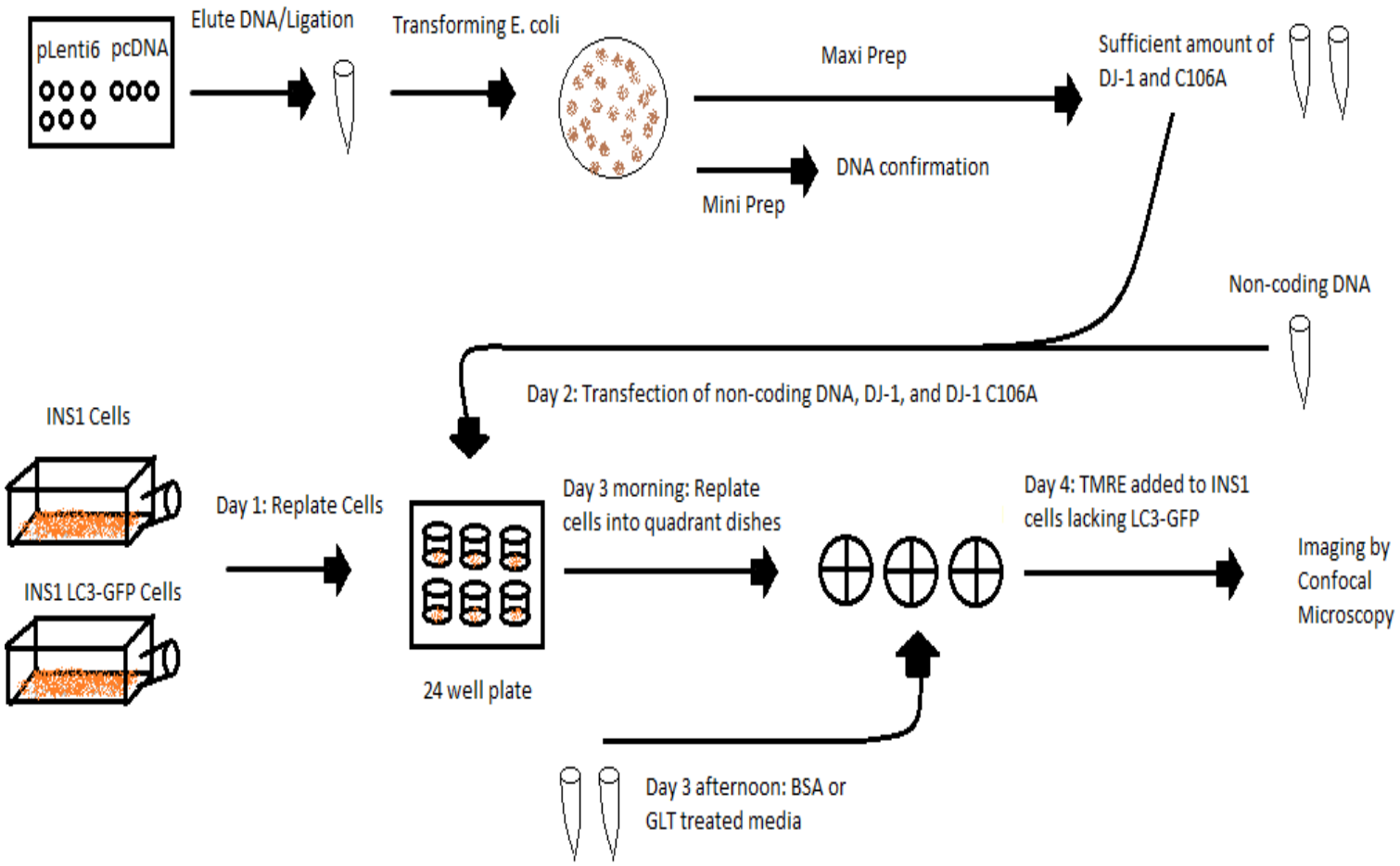

pLenti6 pcDNA
Elute DNA/Ligation
Transforming E. coli
Maxi Prep
Sufficient amount of DJ-1 and C106A
DNA confirmation
Mini Prep
Non-coding DNA
Day 2: Transfection of non-coding DNA, DJ-1, and DJ-1 C106A
INS1 Cells
INS1 LC3-GFP Cells
Day 1: Replate Cells
24 well plate
Day 3 morning: Replate cells into quadrant dishes
Day 4: TMRE added to INS1 cells lacking LC3-GFP
Imaging by Confocal Microscopy
Day 3 afternoon: BSA or GLT treated media

Figure 3: Sequence of steps in the present experiment.

Imaging and Data Analysis

The samples housed in quadrant dishes were imaged using a confocal microscope at a 40x magnification. Images were analyzed using Image J software (National Institutes of Health). An unpaired *t* test was used to validate statistical differences. P = 0.05 was the cut off point for statistical significance.

Making Agar Plates

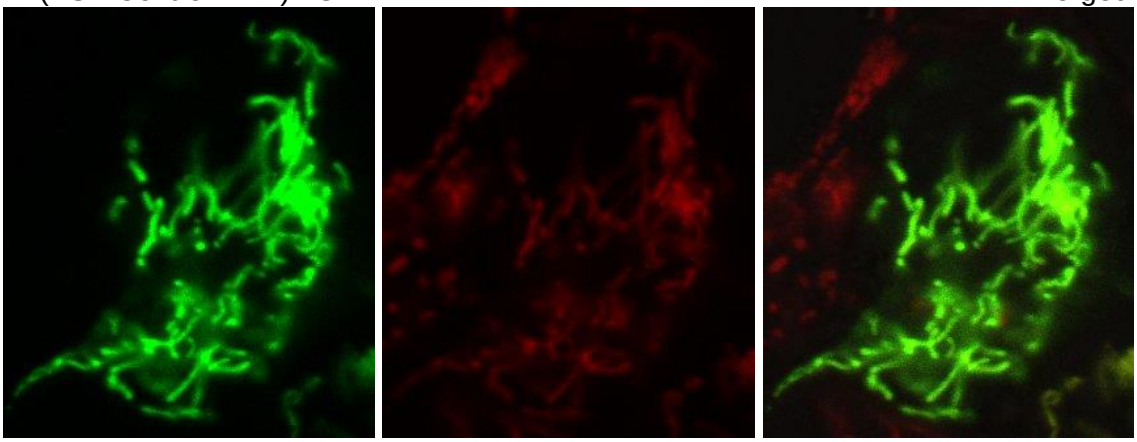
6.4 grams of agar and 200ml of DH20 were autoclaved in an aluminum foil covered 200ml Erlenmeyer flask at cycle setting of Gravity 30 for sixty seven minutes. Following cooling of the mixture, 200µl of ampicillin was added and the subsequent mixture was poured into approximately twenty petri dishes.

RESULTS

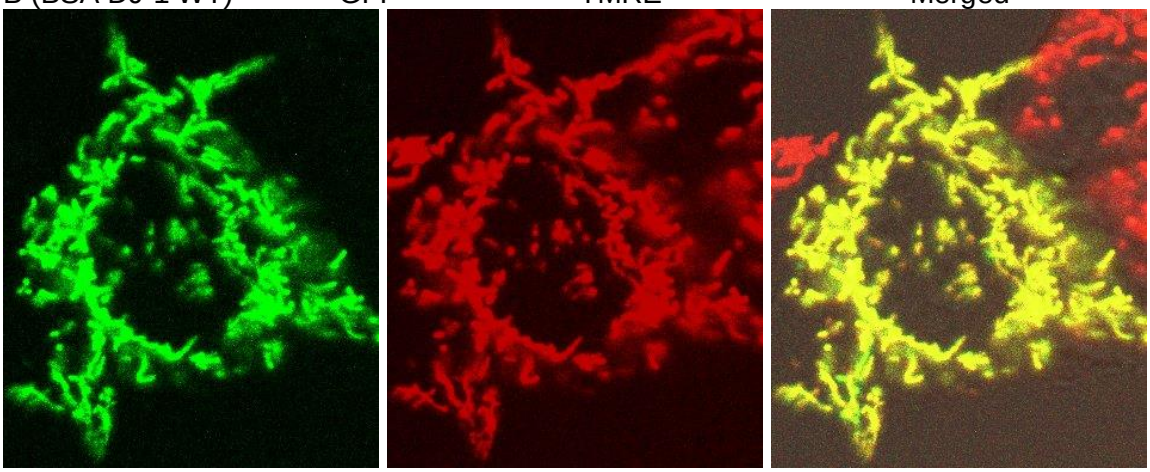
Representative Images

The data collection method for these experiments was dependent on the imaging of cells at multiple wavelengths of color or florescence intensity. Thus, when the laser of the confocal microscope was activated, the mitochondria chosen for imaging were those that appeared yellow. A cell with yellow mitochondria showed that both the DNA bound to GFP had been transfected and that the TMRE dye had accumulated in the mitochondrial population. By analyzing these images in either the green, red, or merged form, we were able to discern the following data (Figure 4).

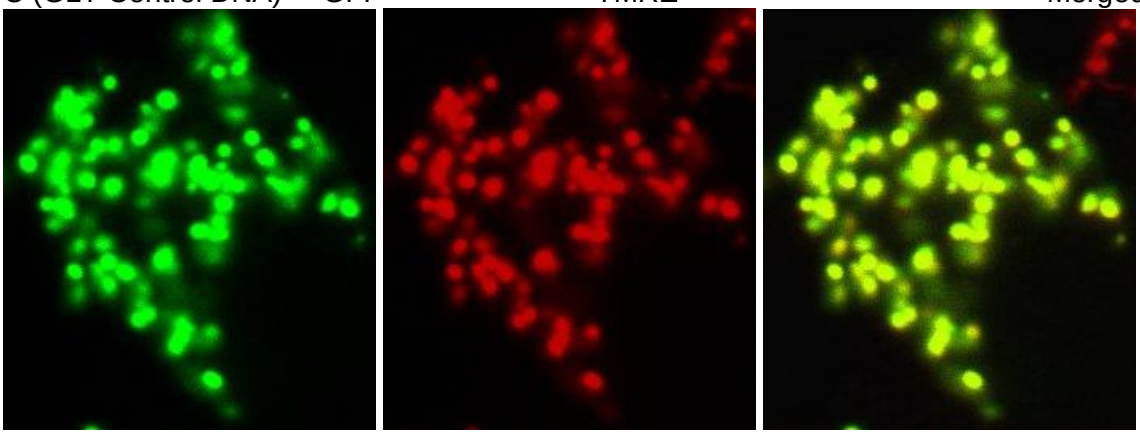
A (BSA Control DNA) GFP TMRE Merged



B (BSA DJ-1 WT) GFP TMRE Merged



C (GLT Control DNA) GFP TMRE Merged



D (GLT DJ-1WT) GFP TMRE Merged

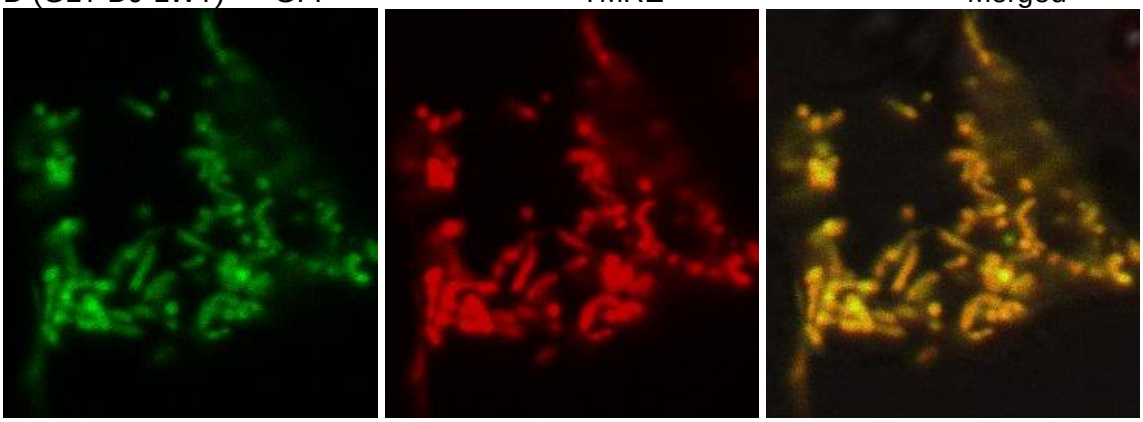
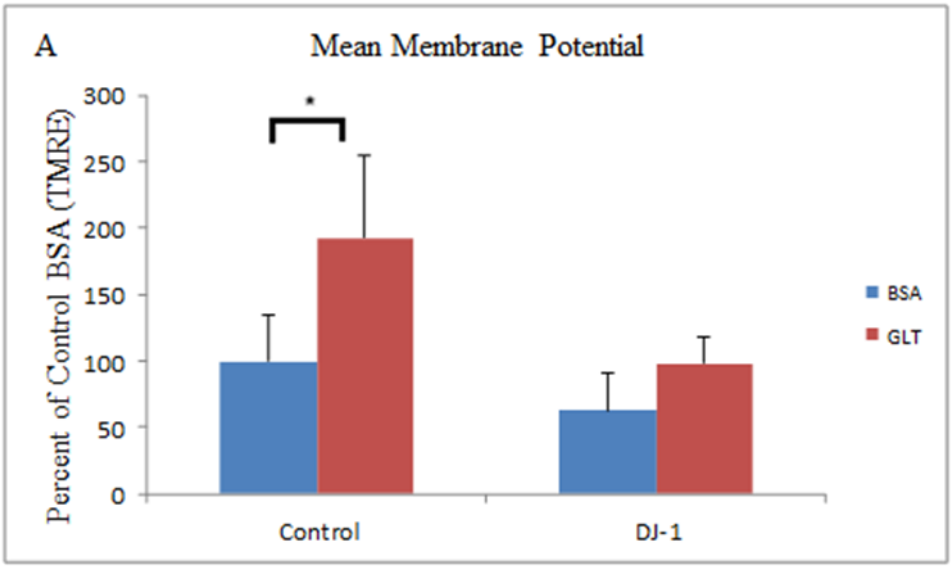


Figure 4: Representative images of 4 INS1 treatment groups. Only experimental treatment groups from successful GLT treatment experiment are included.

Mean Membrane Potential and Heterogeneity Results

Measurements of $\Delta\Psi_m$ were found by analyzing the ratio of TMRE to mitochondrial GFP. For the control DNA group, the GLT treatment yielded a $\Delta\Psi_m$ that was 192.87% of the BSA control (P = 0.0156). In addition, for DJ-1 WT group of cells, the GLT treatment yielded a $\Delta\Psi_m$ that was 98.57% of that of the BSA control while the BSA treatment for the DJ-1 WT group only showed 63.42% (P = 0.0215)(Figure 5A, n = 5-8).

In a repetition of the experiment, an inefficient GLT treatment yielded a non-significant increase in $\Delta\Psi_m$ that was only 104.99% of the BSA control. Due to this inefficiency, the values for other variables tested in this experiment such as heterogeneity and morphology did not merit reporting in this document. The values for $\Delta\Psi_m$ though, shed light on the fact that the GLT treated DJ-1 WT and the DJ-1 C106A expressing groups both showed a significant decrease in $\Delta\Psi_m$ and were 65.08% and 66.13% of the level of the BSA control respectively (P = 0.0009, P = 0.0196). In addition, these two measurements were not significantly different from each other (P = 0.1051) (Figure 5B, n = 4-6).



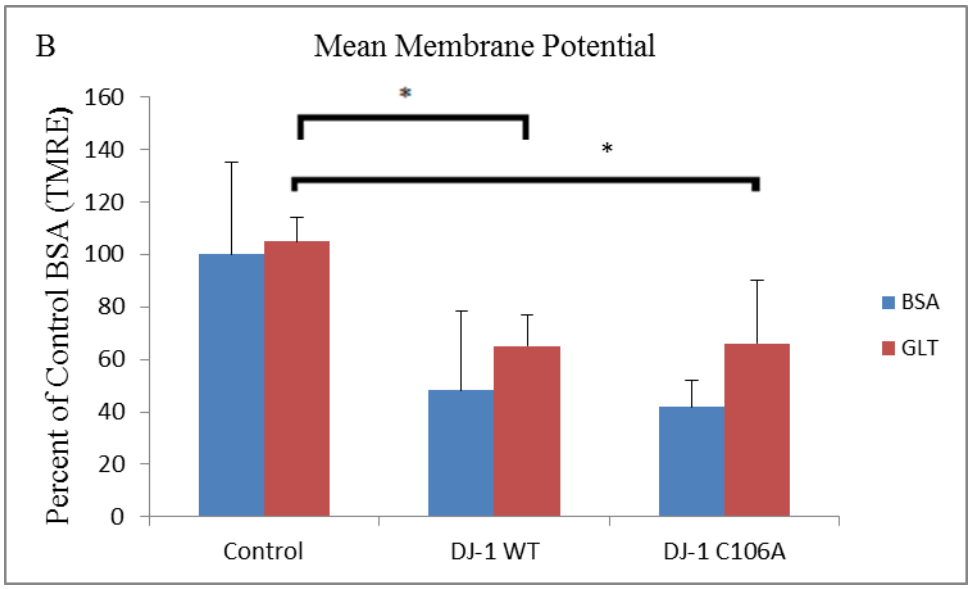
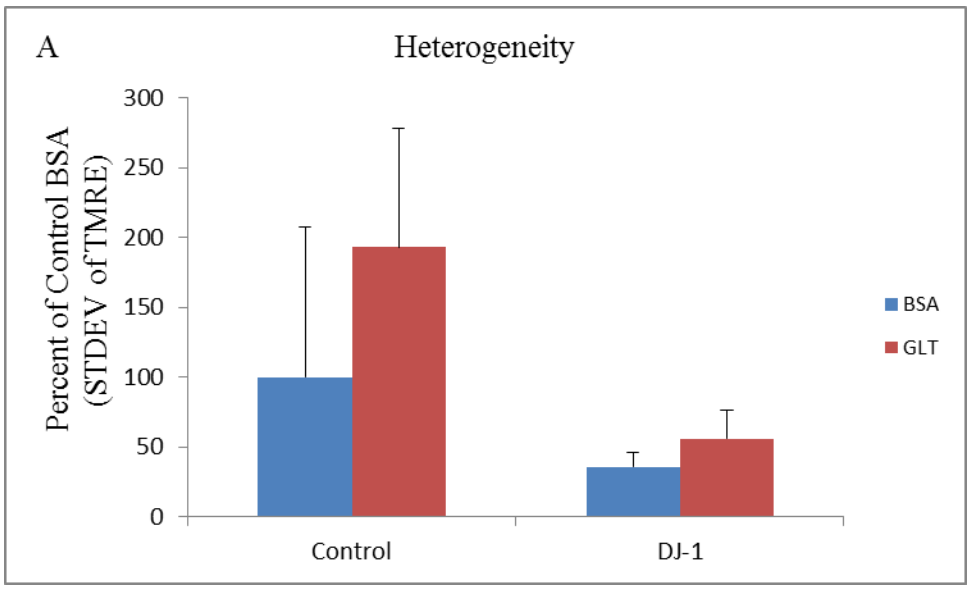


Figure 5: Assessment of $\Delta\Psi_m$ using TMRE. (A) Highly efficient GLT treatment. (B) Inefficient GLT treatment. Error bars represent standard deviations and all values are expressed as a percentage of the control BSA experimental group.

Heterogeneity of the $\Delta\Psi_m$ was found by analyzing the raw standard deviations of the TMRE reading for $\Delta\Psi_m$. In addition, the GLT treatment resulted in a level of heterogeneity 192.86% as great as that of the BSA control, but this was not significant due to the large standard deviation between the two. It is noteworthy to state that there was nearly a significant difference between the BSA and GLT treated cells expressing DJ-1 (P = 0.0649)(Figure 6A, n=5-8).

In a repetition of the experiment, an inefficient GLT treatment yielded a non-significant decrease in content heterogeneity that was merely 92.54% of the BSA control. The GLT treated DJ-1 WT expressing group showed an increase in content heterogeneity and was 101.49% as great as the BSA control value. (Figure 6B, n = 4-6).



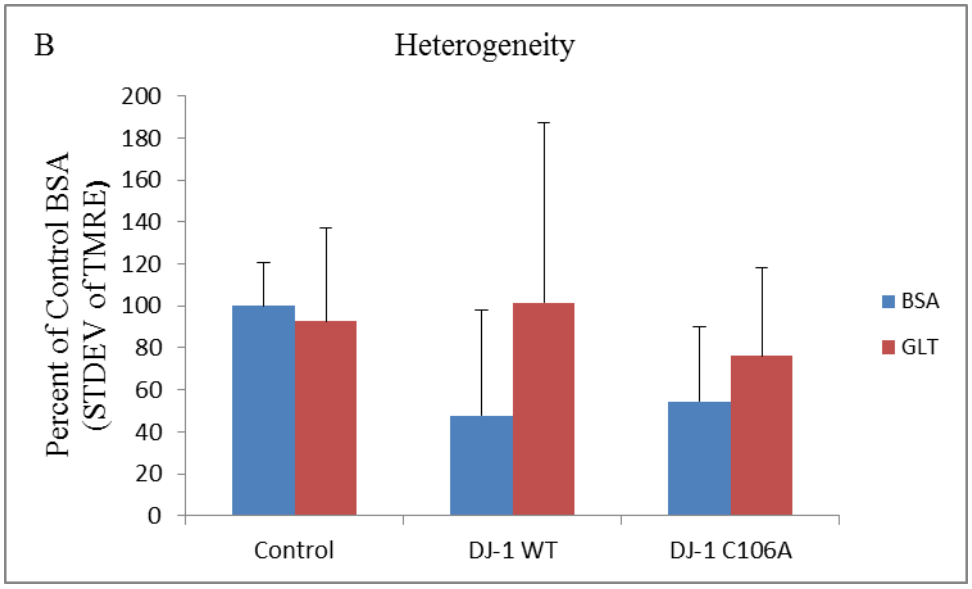


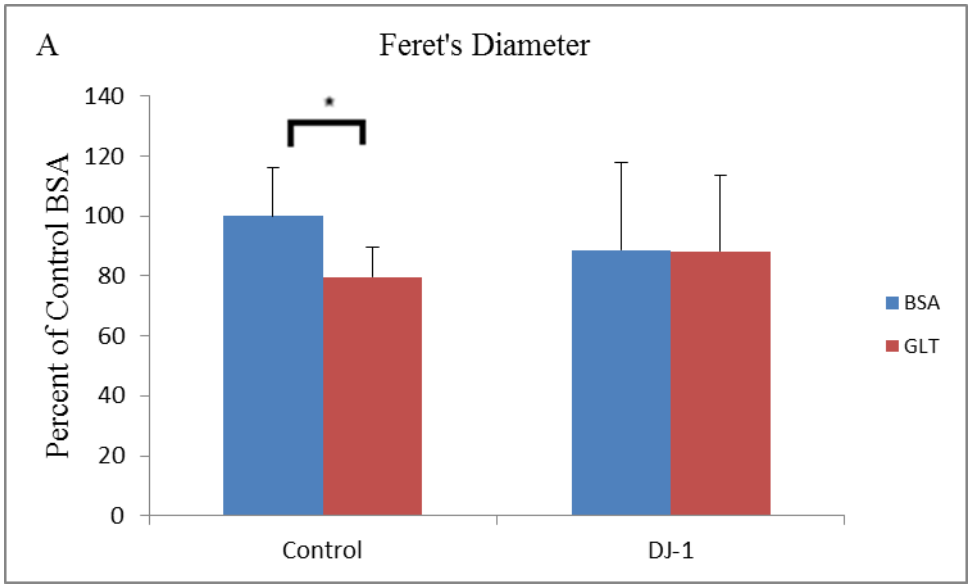
Figure 6: Assessment of mitochondrial content heterogeneity. This was measured by using the standard deviation of $\Delta\Psi_m$ in B-cells. (A) Highly efficient GLT treatment. (B) Inefficient GLT treatment. Error bars represent standard deviations and all values are expressed as a percentage of the control BSA experimental group.

Morphology Results

Feret's diameter was one tool we used to measure mitochondrial morphology. The GLT treatment caused a Feret's diameter measurement of 79.58% of the control BSA group (P = 0.0331). For cells expressing DJ-1,there was no significant difference between the Feret's diameter of the cells treated with BSA compared to GLT. The fact that there is no significant change between

the two DJ-1 groups while there is a significant change with the two control groups says that DJ-1 prevents this significant change. (Figure 7A, n = 4-6).

In a repetition of the experiment, an inefficient GLT treatment yielded a non-significant increase in ferret's diameter that was 114.89% of that of the BSA control. The DJ-1 WT expressing cells showed a significant increase in Feret's diameter, measuring 165.65% of that of the BSA control (P = 0.0414). The DJ-1 C106A expressing group showed a slight decrease in Feret's diameter, measuring 101.53% of that of the BSA control (Figure 7B, n = 4-6).



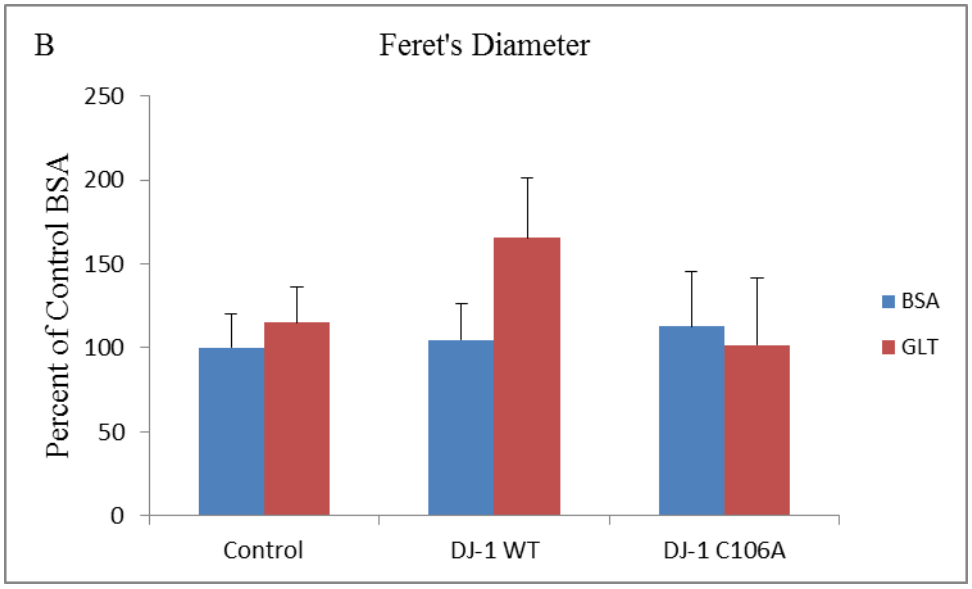
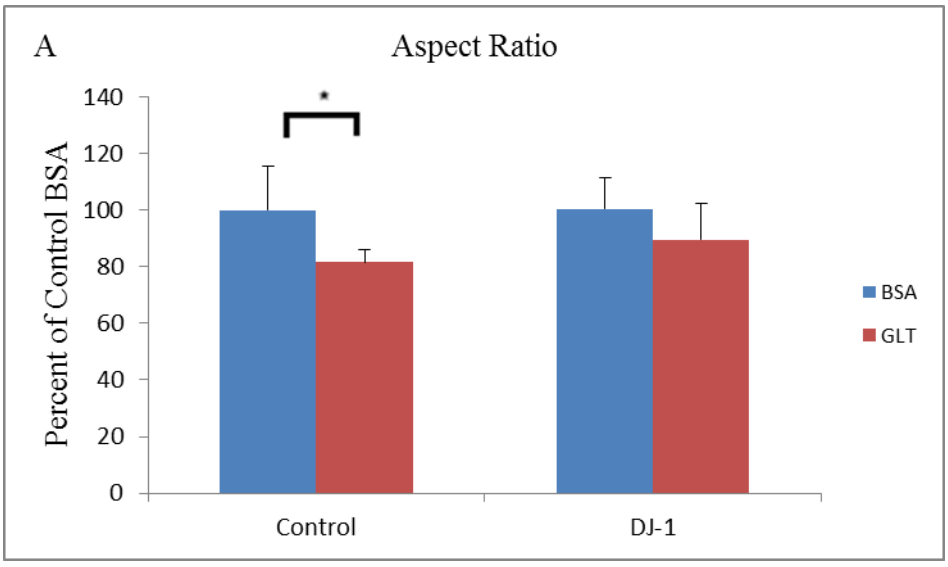


Figure 7: Assessment of DJ-1's potential in altering mitochondrial morphology in pancreatic B-cells by measuring Feret's diameter. (A) Highly efficient GLT treatment. (B) Inefficient GLT treatment. Error bars represent standard deviations and all values are expressed as a percentage of the control BSA experimental group.

Another way we measured mitochondrial morphology was by assessing aspect ratio. The GLT treatment yielded cells with an aspect ratio 81.68% as great as that of the control BSA group (P = 0.0188). The GLT treated cells expressing DJ-1 WT did not show a significant reversal of the previous effect, only showing an aspect ratio measurement of 89.60% of the control BSA group's measurement (Figure 8A, n = 5-8).

In a repetition of the experiment, an inefficient GLT treatment yielded a non-significant decrease in aspect ratio that was 94.21% of that of the BSA control. In addition, the DJ-1 WT expressing cells also showed a decrease in aspect ratio, measuring 93.16% of the control BSA group. (Figure 8B, n = 4-6).



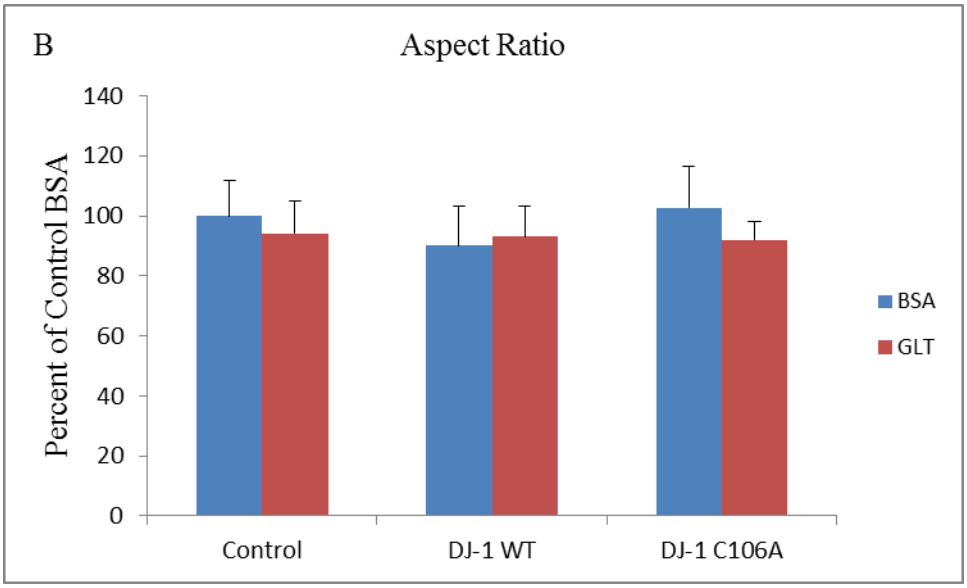


Figure 8: Assessment of DJ-1 potential in altering mitochondrial morphology in pancreatic B-cells by measuring aspect ratio. Error bars represent standard deviations and all values are expressed as a percentage of the control BSA experimental group.

Form factor was another tool we used to measure mitochondrial morphology. The GLT treatment showed cells with a form factor 79.75% as great as the form factor of the control BSA group. This was a significant decrease in form factor ($P = 0.0346$). In addition, for the cells expressing DJ-1, there was not a significant difference between the BSA and GLT treated groups ($P = 0.5484$) (Figure 9A, n = 5-8).

In a repetition of the experiment, an inefficient GLT treatment yielded a non-significant decrease in form factor measuring 90.17% of that of the control BSA group. In addition, the DJ-1 WT expressing cells showed an increase in form factor measuring 105.78% of that of the control BSA group (Figure 9B, n = 4-6).

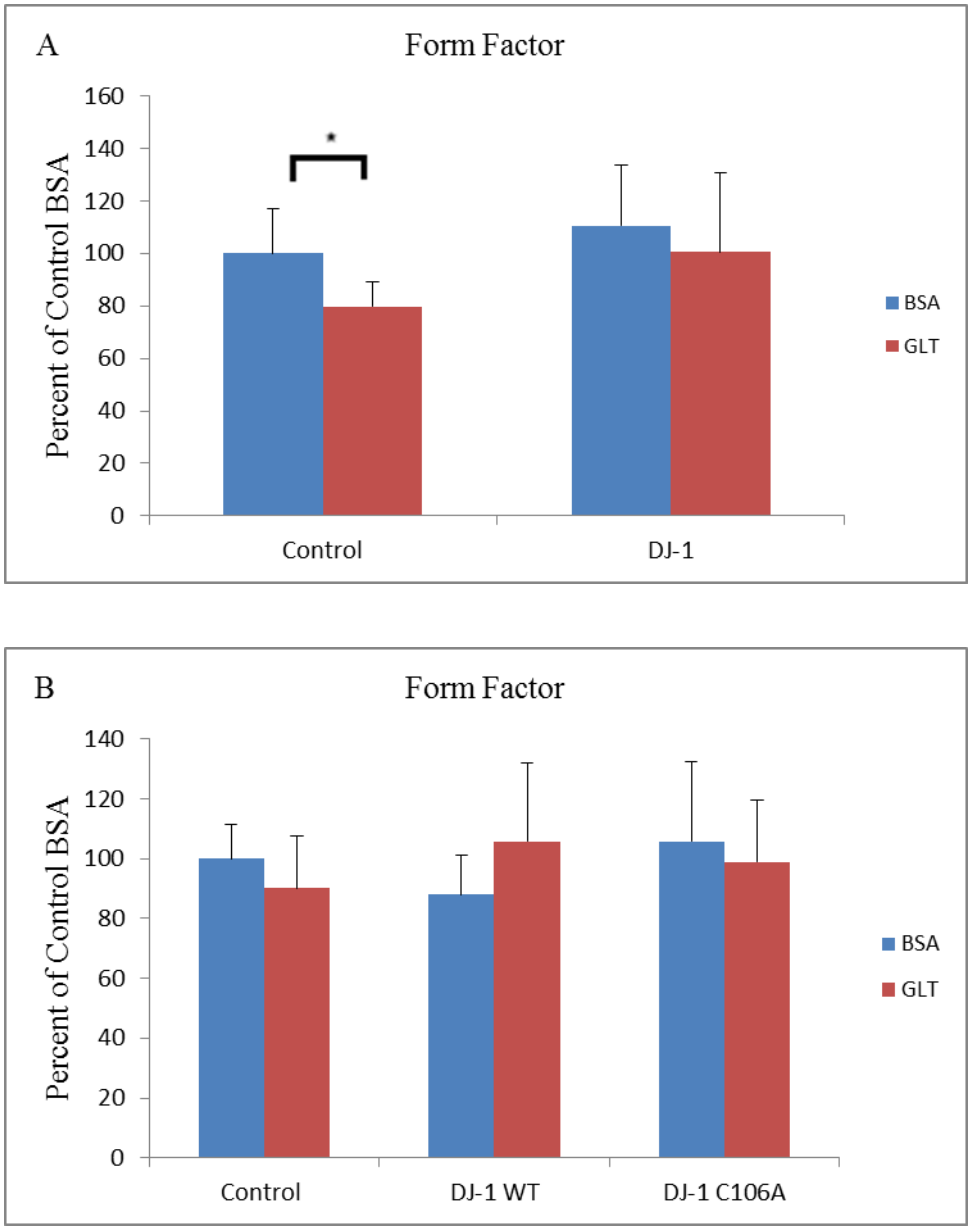
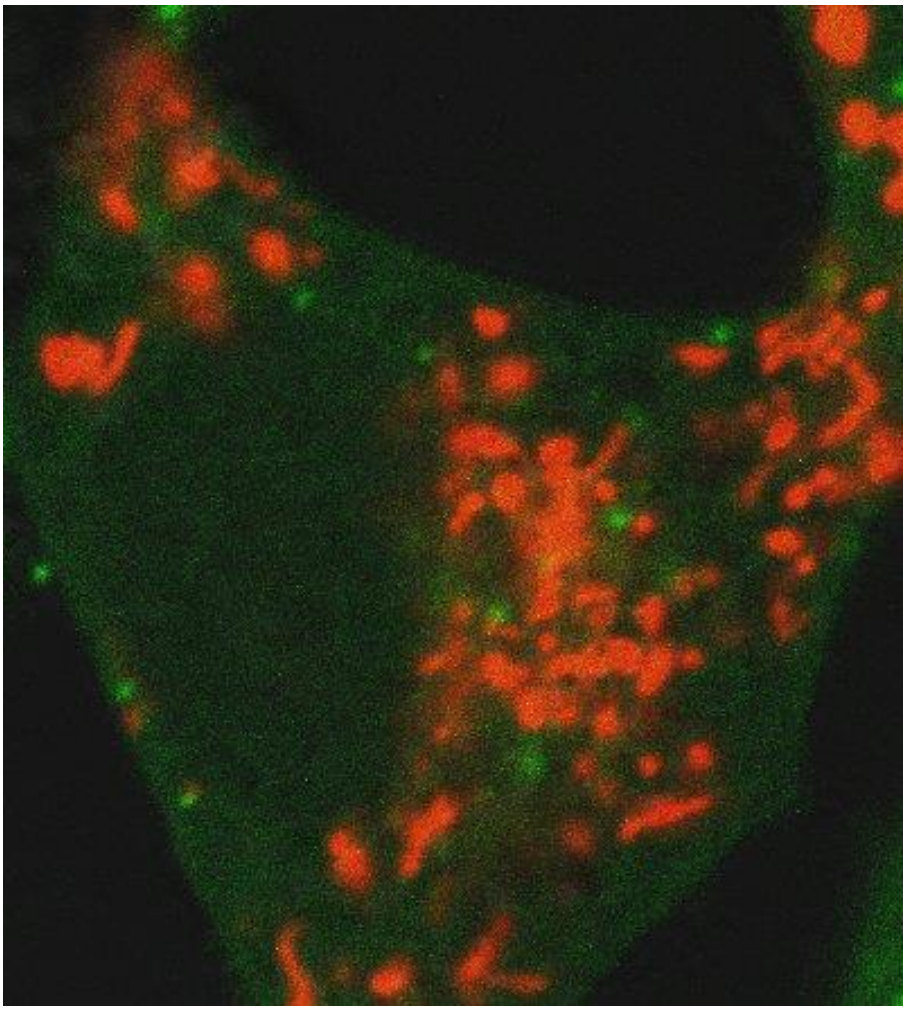


Figure 9: Quantification of DJ-1 potential in altering mitochondrial morphology in pancreatic B-cells by measuring form factor. Error bars represent standard deviations and all values are expressed as a percentage of the control BSA experimental group.

Autophagosome Result

BSA Con Merged



BSA DJ-1 Merged

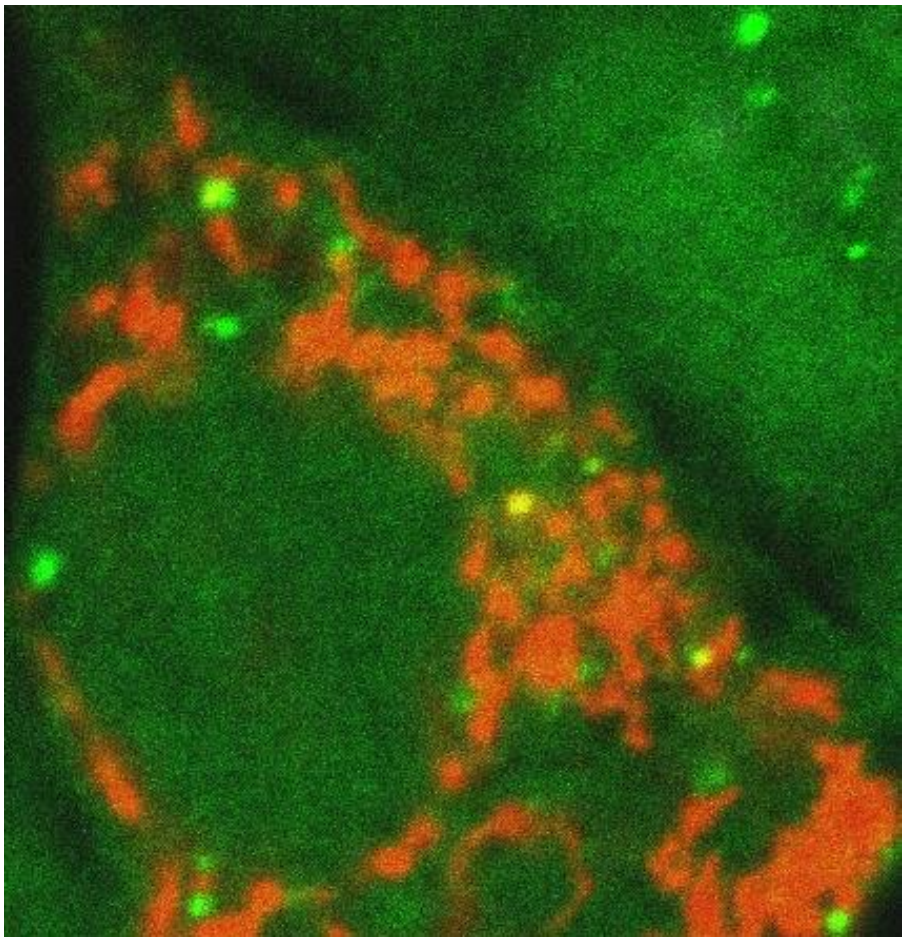


Figure 10: Representative images of the merged BSA Con and DJ-1 Con.

For the cells expressing DJ-1 WT, DJ-1 was able to significantly decreased the average number of APs from 84.38% of the BSA control to 62.87% (P = 0.014)(Figure 11).

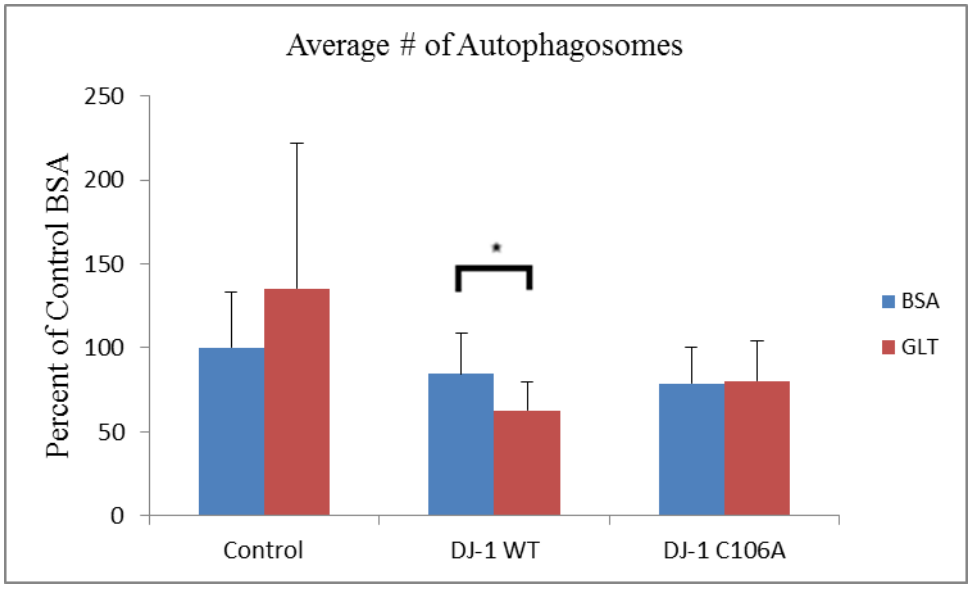


Figure 11: Assessment of average number of autophagosomes.

DISCUSSION

This study deals with the potential of the gene DJ-1 to act as a sensor for oxidative stress in order to enhance mitochondrial quality control. A mitochondrial model for glucolipotocity in INS1 cells was used to study type 2 diabetes.

The Image J software supplied a means to assess mitochondrial morphology in terms of three variables: Feret's diameter, aspect ratio, and form factor. All three of these morphology measurements showed the same effect. The GLT treatment was able to significantly increase all three measurements in cells that were not expressing DJ-1. The absence of a significant decrease in these three measurements for the GLT treated DJ-1 expressing group showed that DJ-1 significantly reverses morphology changes due to glucolipotoxicity. The finding for Feret's diameter, the measurement of the largest line through the mitochondria, agrees with previous studies that say that healthy mitochondria are more likely to be elongated in shape and form networks with other mitochondria.

This significant effect fits well with our understanding of aspect ratio as well. Since aspect ratio is the measurement of the major axis divided by the minor axis, the lowest value is 1, a perfect circle. Thus, a low value is a good indicator for a fragmented mitochondria. Since during the GLT treatment, the cell is more likely to produce more isolated mitochondria that have accrued oxidative damage, these cells are more likely to be fragmented in shape and so a lower aspect ratio is understandable. Since there was not such a comparative change

in morphology in the DJ-1 expressing cells treated with BSA or GLT, the effect shown through aspect ratio is very informative.

Form factor, the measurement of theoretical area by actual area is an indicator of the level of branching found in a mitochondria such that a perfect circle scores the lowest value, 1. Since there was no significant decrease in form factor in the DJ-1 expressing cells while there was there was in the control GLT treatment, DJ-1 significantly shows a reverse in the morphology change. Together, Feret's diameter, aspect ratio, and form factor make a convincing case for DJ-1's ability to enhance quality control by reversing changes in morphology.

After many failed attempts and continued optimization of the experiment, a significant increase in mitochondrial membrane potential was found in GLT treated cells expressing control DNA. This relationship served to distinguish trustworthy results from nebulous findings in relation to the GLT treatment. While DJ-1 successfully reversed changes in morphology, it was not able to significantly reverse changes in mean membrane potential caused by GLT treatment.

Despite the aforementioned test for validity, data from an inefficient GLT treatment yielded interesting result in the realm of mean membrane potential measurements. Together, both experiments showed that DJ-1 does limit the maximal mean membrane potential reached under the GLT treatment. In addition, this partially GLT treated experiment supports the claim that DJ-1 mediates a decrease in mean membrane potential that does not stem from the

function of the C106A region. This observation shows that there could be a multitude of different active sites on the protein for possible interaction. The mapping of such alternative sites of activity would open up new avenues for modulation of DJ-1 activity and thus hold great potential for eventual clinical use.

Our data for heterogeneity of the mitochondrial population did not yield any significant results. Still though, the increase found in the GLT group compared to the BSA group for the cells expressing DJ-1just barely missed being a significant change (P = 0.0649). This trend could easily translate to a significant effect in heterogeneity if the sample size was increased. An increase in heterogeneity would fit in well with our understanding of DJ-1 function. Such an increase would mean that mitochondria which consolidate damaged materials are cleared slower, and so the membrane potential in the mitochondrial population shows more heterogeneity. The GLT treatment has been shown to promote complementation between the glucose and palmitate, yielding a significant decrease in autophagic flux (Las et al 2011). This was originally found through the connection between glucolipotoxicity induced cell death and buildup of APs (Las et al 2011). Thus future studies should quantify GLT induced cell.

We performed the same GLT based DJ-1 experiment using INS1 cells expressing GFP-LC3. The addition of the GLT treatment to cells expressing DJ-1 showed a significant decrease in the average number of autophagosomes present in the cells. This can either be due to increased clearance through autophagy or a decreased production of autophagosome.

Future studies involving DJ-1's potential in enhancing quality control would incorporate cell viability measurement and oxygen consumption in order to better understand DJ-1's ultimate effects on pancreatic B-cells.

JOURNAL ABBREVIATIONS

Abbreviation Full Name

Adv Exp Med Biol- Advanced Experimental Medical Biology

Curr Opin Neurobiol. – Current Opinions in Neurobiology

Int J Biochem Cell Biol – International Journal of Biochemistry and Cell Biology

Hum Molec Genet – Human Molecular Genetics

J Biol Chem- Journal of Biological Chemistry

J Bioenerg Biomembr- Journal of Bioenergetics and Biomembranes

Philos Trans R Soc Lond B Biol Sci. – Philosophical Transactions of the Royal Society of London, Series B, Biological Sciences

PLOS Medicine – Public Library of Science

Proc Natl Acad Sci U S A – Proceedings of the National Academy of Sciences

Trends Biochem Sci.- Trends in Biochemical Sciences

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