USING CHANNELRHODOPSIN-2 TO INDUCE APOPTOSIS

A Major Qualifying Project Report
Submitted to the Faculty
Of
WORCESTER POLYTECHNIC INSTITUTE
In partial fulfillment of the requirements for the
Degree of Bachelor of Science

Amanda Sargent

Date: April 28, 2011

Approved

Robert Dempski, Advisor

John MacDonald, Advisor

Abstract

This project will focus on using channelrhodopsin-2, a light activated ion channel, to induce apoptosis, programmed cell death. It will begin with the transformation of a plasmid containing a fusion protein including channelrhodopsin-2 and green fluorescent protein (GFP) along with a signal sequence to direct the fusion protein to the mitochondria of HEK-293 cells. Next it will be necessary to check the cells to make sure the fusion protein is in the mitochondria by comparing it to known probes, such as MitoTracker Deep Red FM using confocal microscopy. Following this, a blue light will be applied to the sample to check for calcium influx which will indicate that cytochrome C has been released from the inner membrane of the mitochondria. Cytochrome c release is the first committed step in apoptosis.

Table of Contents

Introduction and Background

Channelrhodopsins are a family of proteins that function as light-gated ion channels.[1] Channelrhodopsin-2 is an algal protein that offers new and promising solutions to the treatment of diseases such as Parkinson's by permitting minimally invasive, genetically targeted and temporarily precise photostimulation. Channelrhodopsin-2 (ChR2) is expressed mainly in C. reinhardtii under low-light conditions according to research done by Peter Hegemann and his lab.[2] ChR2 has seven transmembrane domains. The retinal chromophore is covalently linked to the rest of the protein through a lysine residue by a Schiff base which is protonated. The C-terminal end of the protein extends into the intracellular space and can be replaced by fluorescent proteins without affecting channel function.[3] In the presence of a blue light, a ChR2 channel opens as can be seen in figure 1. Channelrhodopsins can be readily expressed in excitable cells such as neurons using stable transfection with HEK293-Trex cells.[4]

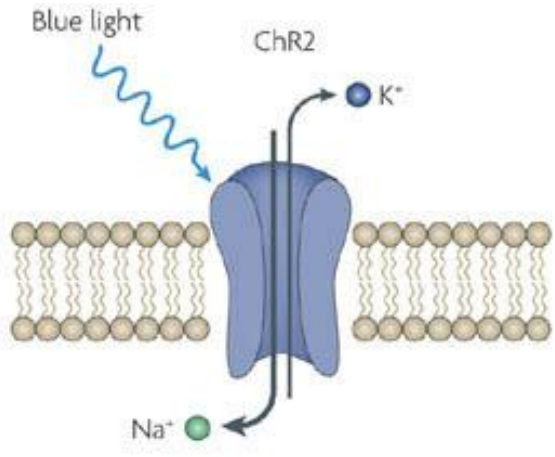

Figure 1. Opening of ChR2 in Presence of Blue Light5

[1]Nagel, G, Szellas T, Kateriya S, Adeishvili N, Berthold F, Ollig D, Hegemann F, Bamberg, Ernst. (November 2003) "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel" PNAS November 25, 2003 vol. 100 (24) :13940-45

[2] Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci USA 100 13940–13945

[3] Nagel, G, Szellas T, Kateriya S, Adeishvili N, Berthold F, Ollig D, Hegemann F, Bamberg, Ernst. (November 2003) "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel" PNAS November 25, 2003 vol. 100 (24) :13940-45

[4] Fieldbauer, Katrin, Zimmerman, Dirk, Pintschovius, Verena, Spitz, Julia, Bamann, Christian, Bamberg, Ernst. (July 2009) "Channelrhodopsin-2 is a leaky proton pump" PNAS July 28, 2009 vol. 106 no. 30 12317-12322

[5] Zhang, F. et al. Circuit-breakers: optical technologies for probing neural signals and systems. Nature Reviews Neuroscience 8, 577-581 (August 2007)

Channelrhodopsin-2 has become a powerful tool for a variety of purposes. For example, in 2005 Karl Deisseroth and colleagues began using different proteins from the "opsin" family and inserted them into neurons using a virus.[6] Most of the "opsin" families of proteins are ion pumps such as bacteriorhodopsin. ChR2 is used because it is an ion channel.[7] The difference between an ion channel and an ion pump is channels let selected ions diffuse rapidly down electrical and concentration gradients, whereas ion pumps consume energy to slowly move ions against the chemo electric gradient.[8] ChR2 uses energy derived from light to initiate ion conductance. The absorption of a photon of light causes an isomerization of retinal which is a co-factor. This induces a conformational change in the trans-membrane domains of the protein. This opens the channel and allows the movement of ions as seen in figure 2.[9] Using this protein as a tool to control cellular function can be important because this technique could be used in the treatment of diseases such as Parkinson's disease. This can be done through deep brain stimulation, which is a surgical treatment involving the implantation of a brain pacemaker, which sends electrical impulses to specific regions of the brain. If successful, it could also be used in providing insight on autism, schizophrenia, drug abuse, and depression.[10]

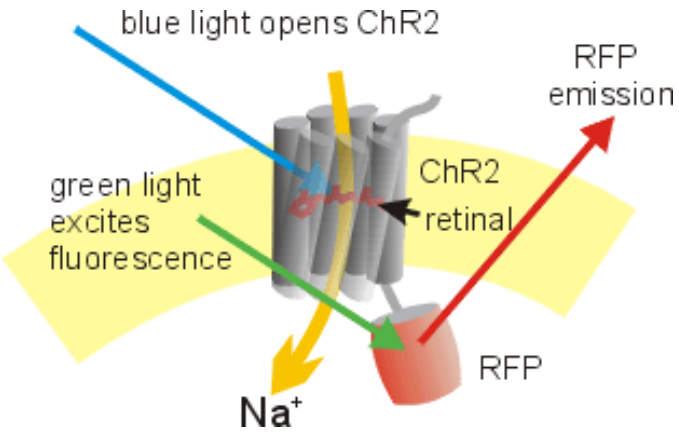

Figure 2. Diagram of Proteins of ChR2

[6]Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (September 2005). "Millisecond-timescale genetically targeted optical control of neural activity". Nat. Neurosci. 8 (9): 1263–8.doi:10.1038/nn152510.1038/nn1525. PMID 1611644

[7] Zhang, Feng et al. Channelrhodopsin-2 and optical control of excitable cells. Nature Methods 3.10, 785-795 (October 2006)

[8] Gadsby, David C. (May 2010) "Ion Channels versus ion pumps: the principle difference , in principle" Molecular Cell Biology 2010 May 1 10(5) 344-352

[9]Kim JM, Hwa J, Garriga P, Reeves PJ, RajBhandary UL, Khorana HG (February 2005). "Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops." Biochemistry 44 (7): 2284–92. doi:10.1021/bi048328i. PMID 15709741.

[10]Zhang, Feng et al. Channelrhodopsin-2 and optical control of excitable cells. Nature Methods 3.10, 785-795 (October 2006)

Channelrhodopsin-2 is currently being used at MIT by Professor Ed Boyden in the treatment of retinitis pigmentosa, which is an inherited form of blindness.[11] They are using ChR2 because the gene for the protein can be modified so that it is only expressed in specific types of neurons. When the gene is delivered to the brain or eyes via gene therapy, the protein expresses to the plasma membrane and opens when exposed to light. Positively charged ions rush into the cell triggering an electrical message. Channelrhodopsin and similar tools could be used in research with diseases such as depression, addiction, and epilepsy.[12] In 2010, Boyden published a paper, stating that "Because the molecule comes from algae, we need to test it in an animal more closely related to humans to see if it triggers an immune response."[13] It is scientist's ultimate goal to use channelrhodopsin to target different cell types that could treat a broad range of diseases.

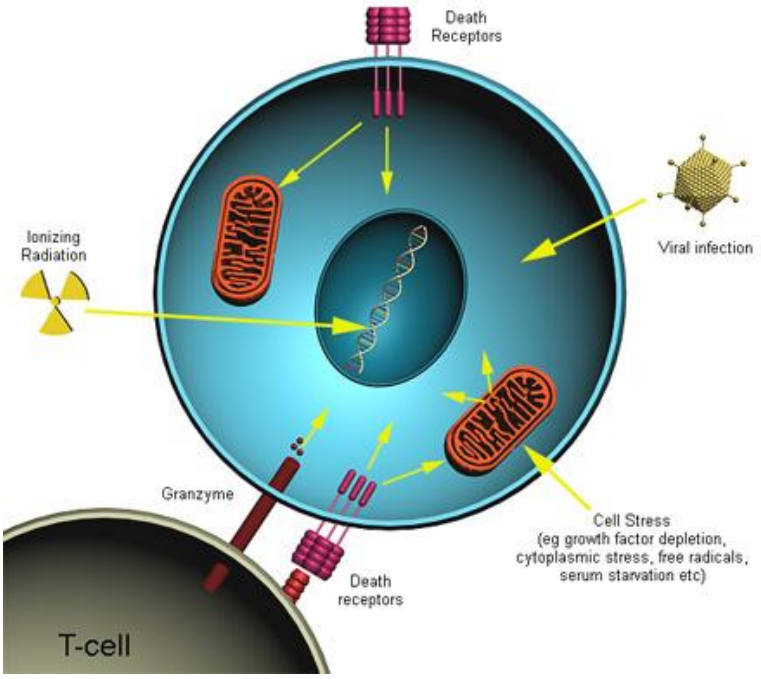

Figure 3. Diagram showing steps of Apoptosis[14]

[11] Zorzos, A. N., Boyden, E. S.*, and Fonstad, C. G. (2010) A Multi-Waveguide Implantable Probe for Light Delivery to Sets of Distributed Brain Targets, Optics Letters 35(24):4133-5.
[12] Zorzos, A. N., Boyden, E. S.*, and Fonstad, C. G. (2010) A Multi-Waveguide Implantable Probe for Light Delivery to Sets of Distributed Brain Targets, Optics Letters 35(24):4133-5.
[13] ibid
[14] Dash, Phil. Apoptosis. Reproductive and Cardiovascular Disease Research Group. http://www.sgul.ac.uk/depts/immunology/~dash/apoptosis/

Using channelrhodopsin-2, which has been known to control calcium influx[15], induction of apoptosis will be attempted. Apoptosis is programmed cell death as seen in figure 3. Apoptosis is different from necrosis which is uncontrolled cell death. Necrosis leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide).[16] Apoptosis can occur when there is an influx on calcium within the cell. This can be induced by ChR2. After the influx of calcium, cytochrome C is released which is the first step in apoptosis.

This project will focus on using channelrhodopsin-2 to induce apoptosis. This will be done by transfecting HEK 293 cells with a fusion protein which is comprised of ChR2, mitochondria targeting primers, and YFP. It is evident that this project could prove valuable to many research scientists studying the same protein as well apoptosis.

Materials and Methods

Transformation of E. coli Cells

Two plasmids were obtained from the laboratory with the vector pcDNA (3.1)- . The first plasmid contained channelrhodopsin-2 with GFP and the sequence KDEL on the C-terminus. The second plasmid was pcDNA (3.1)-, channelrhodopsin-2 with GFP, and the sequence KCPL on the C-terminus. The first step in the process was to transform cells. This was done by using 20 µL of top 10 electro competent cells and 1 µL of plasmid DNA (200ng/µl). Approximately 20 µL of this mixture was put into the pre-chilled electroporator in an ice-water slurry. The electroporator (Cell Porator by Life Technologies) was charged to approximately 430v and the cells were electroporated. The electroporated cells were placed into approximately 1 mL of SOC media (20 g tryptone, 5 g yeast, 0.5 g NaCl, 0.2 g KCl, 1 L ddH_2O, 10 mL 2M Mg^{++}, and 10 mL 2 M glucose) and placed in the incubator with shaking at 37ºC for one and a half hours. After that time approximately 30 µL was plated on a plate containing LB (10 g tryptone, 5 g yeast, and

[15] Schoenengerger, P, Scharer, Y, Oertner, T. (2010) Channelrhodopsin as a tool to investigate synaptic transmission and plasticity, Exp Physiol June 18, 2010
[16] Dash, Phil. Apoptosis. Reproductive and Cardiovascular Disease Research Group. http://www.sgul.ac.uk/depts/immunology/~dash/apoptosis/

10 g NaCl), 15 g Agar, and ampicillin (1ng/mL). The plates were placed in the incubator at 37ºC over night to allow colonies to grow.

Inoculation of Cells

The following day, colonies were observed on the plates and inoculated. Approximately 5 ml of LB/Amp broth (10 g tryptone, 5 g yeast, and 10 g NaCl) were placed into test tubes. All of this was done by the flame in order to keep the atmosphere as sterile as possible. The tubes were then placed in the incubator with shaking at 37°C over night.

Mini-Prep

A 1.5-5 mL aliquot of overnight culture was centrifuged for one minute at a speed of 11,000 x g to pellet cells. The supernatant was then discarded. A mini-prep was performed to obtain plasmid DNA using the Nucleospin© Plasmid Kit (Macherey Nagel). In brief, a 250 µL aliquot of buffer A1 (stored in the 5°C refrigerator) was added. The sample was vortexed to resuspend the cells. Then 250 µL buffer A2 was added. In order to make sure there are no clumps left, the tube was shaken gently 6-8 times. At this point it is important not to vortex the sample to avoid damaging the DNA. The sample was incubated for up to 5 minutes at room temperature or until lysate appeared clear. Then 300 µL of buffer A3 was added. The samples were shaken approximately 6-8 times and subsequently centrifuged for 5 minutes at 11,000 x g. A 750 µL aliquot of each sample was placed into a Nucleospin© Extract II column. The sample was centrifuged for 1 minute, and then the supernatant was discarded. Then 600 µL of buffer A4 was added and centrifuged for 1 minute, discard supernatant. Centrifuge for two minutes to dry the sample and remove any excess liquid. Add 15-50 µL of elution buffer and incubate for up to one minute at room temperature. Centrifuge sample for 1 minute to elute the DNA.

Polymerase Chain Reaction (PCR)

A PCR reaction was set up to incorporate a signal sequence to localize the fusion protein to the mitochondria.[17] The sequence can be seen below. The reactions were run according to the following protocol:

[17] Omura, Tsuneo. (February 1998) "Mitochondria-Targeting Sequence, a Multi-Role Sorting Sequence Recognized at all Steps of Protein Import into Mitochondria." Journal of Biochemistry 123(1010-1016)

Step 1	95°C for 1 minute,
Step 2	95° C for 30 seconds
Step 3	50° for 30 seconds
Step 4	72 ° for 2 minutes
Step 5	Repeat steps 2-4 for 40 cycles.
Step 6	72 ° for 10 minutes
Step 7	4° for up to 24 hours

Table 1. Reaction Settings for PCR

	Reaction 1 (µL)	Reaction 2 (µL)	Reaction 3 (µL)	Reaction 4 (µL)
Distilled H_20	40.1	40.1	40.1	40.1
10x Buffer	5	5	5	5
dNTPs (25nM each NTP)	0.4	0.4	0.4	0.4
DNA template (100 ng/µL)	1	1	1	1
Primer MDH (100 ng/µL)	1.25	1.25	1.25	1.25
Primer #2 (100 ng/µL)	1.25	1.25	1.25	1.25
Cloned Pfu DNA Polymerase (2.5 U/µL)	1	1	1	1
Total Volume	50	50	50	50

Table 2. Protocol for PCR Reaction

The primer sequences are as follows:
Primer MDH:
CTAGTGGATCC**ATGCTCTCCGCCCTTGCCAGGCCGGCGGGGGCGGCGCTGCGG CGG****GATTATGGAGGCGCCCTG
Primer #2: AACTTAAGCTTTTACTTGTACAGCTCGTCCATGCC

** Mitochondria targeting sequence denoted in bold

The sequences are based off of mitochondria targeting sequences from the research of Tsuneo Omura.[18] To determine if there was a PCR product, the sample was checked on an agarose gel for 30 minutes.

Digest

After the PCR is completed, both the parent plasmid and vector were digested prior to ligation. For this experiment 20 µL of plasmid/DNA (200ng/µL) were added to a 1.5 ml centrifuge tube. Also add 3 µL of Buffer 2, 3 µL of BSA (10x), 2 µL of ddH_20, 1 µL of EcoRV (New England Bio Labs) and 1 µL of HindIII for a combined reaction volume of 30 µL. Let the reaction sit in the water bath at 37°C for one and a half hours.

DNA Extraction from an Agarose Gel

A low melt agarose gel was poured to isolate the digested DNA. To do this, combine 0.5 g of low-melt agarose (Ibisci) and combine with 50 mL of 1x TAE buffer (121g Tris base in 250mL, ddH2O, 28.6mL acetic acid, 50mL 0.5M EDTA pH 8.0). Microwave for approximately 45s but be careful not to let it boil over. In order to prevent it from boiling over place a Kim wipe at the top of the flask. Then add 50 µL of 1000x ethidium bromide in the hood. Swirl the sample and pour into the gel plate. The gel ran in 1x TAE buffer at 96 volts for about a half hour. In order to then remove the DNA sample from the gel you must extract the DNA with a scalpel under UV light. Then place the sample into a weighed centrifuge tube. Then weigh the sample. To extract the vector or plasmid from the gel, the Nucleospin© Extract II kit was used. For every 100 mg of sample add 200 µL of buffer NT. Make sure the gel has completely melted by placing it in a water bath at 50° C for 10 minutes. Vortex the sample every 2-3 minutes until it is completely dissolved. Then place the sample in a Nucleospin© Extract II column and centrifuge for 1 minute at 11,000 x g. Discard the supernatant and add 700 µL of buffer NT3. Centrifuge for 1 minute and discard the supernatant. Then centrifuge for 2 minutes in order to remove any excess liquid from the sample and discard and any supernatant. Lastly, add 15-50 µL of buffer NE and centrifuge for 1 minute to elute the DNA.

[18] Omura, Tsuneo. (February 1998) "Mitochondria-Targeting Sequence, a Multi-Role Sorting Sequence Recognized at all Steps of Protein Import into Mitochondria." Journal of Biochemistry 123(1010-1016)

Ligation and Transformation of Cells

For ligation set up the following reaction as listed in the table:

Component	Amount (µL)
5x ligase buffer	4
Vector	3.44
Insert	7.79
dd H_20	3.77
T4 ligase (NEB)	1
Total volume	20

Table 3. Ligation Reaction Protocol

The reaction proceeded at room temperature for 5 minutes and then it was placed on ice to quench. To transform XL-10 Gold Ultra competent cells (Agilent Technologies), chill four centrifuge tubes on ice and preheat 4 tubes of SOC (20g tryptone, 5 g yeast extract, 0.5g 5M NaCl, 0.2g of 1M KCl, 10 ml of 2M Mg^{++}, 10 ml of 2M glucose, and ddH_2O) media in the 37°C water bath. Then thaw the cells. Next, aliquot 30 µL of the sample into each centrifuge tube. Add 2 µL of β-mercaptoethanol. Swirl gently and incubate on ice for 10 minutes, swirling every 2 minutes. Next, add 2 µL of the ligation mixture to cells and swirl gently. Incubate the samples on ice for 30 minutes. Then heat pulse the tubes in a 42°C water bath for **exactly** 30s. Then incubate the cells on ice for 2 minutes. Next, add 0.9 mL of SOC media to each sample and incubate for 1 hour and 45 minutes in the shaker at 37°C. Finally plate 50 µL of each sample on a plate (LB/Amp) and 7 µL of each sample on a plate for a total of 8 plates.

Cell Culture

For cell culture, HEK293 cells were thawed and the media was prepared. During all steps of this process everything was kept sterile and washed with 70% EtOH before entering the hood, including hands. Before beginning make sure all media is properly thawed in a water bath to 37°C, then prepare the media. DMEM is stored at +4ºC; fetal bovine serum is stored at -20ºC as well as penicillin/streptomycin. Combine 10% fetal bovine serum (approximately 50 µL) with 1% Penicillin/streptomycin (approximately 5 µL) in a bottle of DMEM, and then thaw the cells. In order to do this you must first remove the cryo-vial from the -80°C freezer. Then add to 0.5 ml of warm media to the vial with the cells and hold in fingers to thaw rapidly. Next, place 10 ml warm media in 15 mL centrifuge tube. Centrifuge for 5 minutes, and then remove liquid using vacufuge in the hood. Then add another 10 mL of media and centrifuge again for 5 minutes.

Remove media and resuspend cells in 10 ml of media. Plate 5 ml of sample per flask. Incubate cells for 3-5 days.

After three to five days, it is necessary to split the cells before they reach 100% confluency. Pre-warm the DMEM medium, PBS, and trypsin thirty minutes prior to splitting the cells in a 37ºC water bath. After the medium is warmed begin splitting the cells. In order to do this, tip the flask on its side and remove all the media. Then add approximately 2 mL of PBS to the flask. Move the flask from side to side to thoroughly rinse the cells. Then again tip the flask on its side in order to collect the cells and remove the PBS. Next, add approximately 2 mL of trypsin. Repeat the same procedure as with the PBS then remove the media. Place the flask flat in the hood and allow the cells to sit for about two minutes. After two minutes, add 5 ml of DMEM media with serum. Resuspend the cells. Then take approximately 1 ml of the cells in media and place in a new flask with 4 ml of DMEM media. Then allow the cells to incubate for 3-5 days and repeat this process.

Transfection and MitoTracker Staining

For MitoTracker staining, prepare a stock solution in DMSO to a final concentration of 1 mM. Dilute the 1 mM MitoTracker stock solution to a final working concentration of 25, 250, and 500 nM in order to test the best concentration. Be careful not to expose it to light as it is extremely light sensitive. For staining live cells use DMEM medium that has been used to split cells with FBS and penicillin and streptomycin. Add the correct amount of solution to the cells (usually about 1.25 µL) and incubate for 15 minutes at 37º C. Then remove the medium and replace it with fresh DMEM medium. Place back in incubator until you are ready to test under the confocal. Do not let sit longer than a few hours.

One day before transfection, plate cells at a 1:5 ratio in poly-d lysine wells (Mattek) using DMEM medium. Allow to incubate over night at 37º C. The next day, begin the transfection experiment by adding 250 µL of Opti-MEM® I Reduced Serum Medium to a 1.5 µL centrifuge tube. Do this for two centrifuge tubes. To the first centrifuge tube add 12 µL of DNA (0.332 ng/µL), allow to incubate for 5 minutes at room temperature. To the second tube add 10 µL of Lipofectamine™ 2000 (Invitrogen) and allow to incubate for 5 minutes at room temperature. Then add the contents of tube 1 to tube 2. Allow to incubate at room temperature for 20 minutes.

After incubation add approximately 500 µL of the sample to the cells that were split into the well the night before. Incubate for approximately 3.5 hours and then change the medium. Allow to then incubate for approximately 48 hours. Repeat the process the same way except only allow the transfected cells to incubate for 24 hours to test the effect of incubation time on cell life. Use a 500 nM concentration of MitoTracker following the method above to check the samples with the confocal.

Results and Conclusion

Polymerase Chain Reaction and Ligation

The PCR experiment was designed to amplify the DNA sample and to insert the mitochondria targeting sequence in primer MDH into the sample. After the PCR was completed, the samples were checked on a gel for the proper results. The following, figure 4, shows the results from the PCR. As you can see in lanes 2-5 there are bands about half way done the gel around 1800 base pairs, which is where the ChR2 should be. This shows that the sample does contain the ChR2 which is expected in a successful PCR. Lane 1 is the hyperladder.

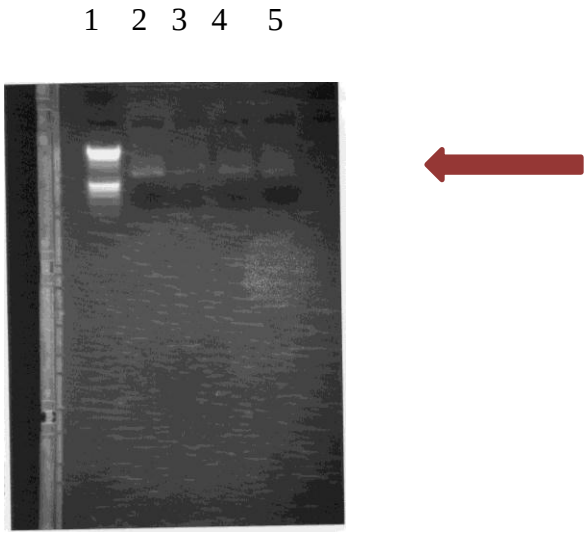

Figure 4. PCR Results showing lanes 1-5, the arrow depicts the ChR2 at 1800 bp

After the PCR was completed, ligation and a digest were performed to check for the presence of the DNA and the plasmid. Figure 5 shows the results from the digest after ligation. Lane 1 shows

the hyperladder, lane 2 shows the presence of the plasmid above the DNA and a very faint band for the DNA. Lane 3 shows no results. Lanes 4 and 5 show both the plasmid and the DNA. This is the expected result after ligation showing that the ligation was successful in fusing the insert and the plasmid together.

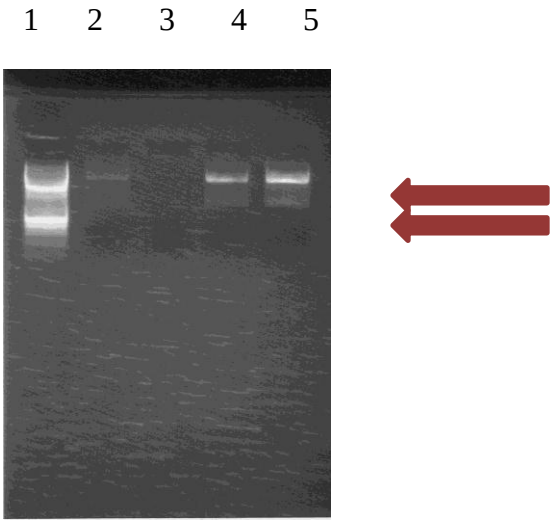

Figure 5. Digest after Ligation Results, the top arrow shows the plasmid and the bottom arrow shows the ChR2 at 1800 bp

Cell Culture, Staining and Transfection

After completing the digest and ligation, the next step was to test the cell culture experiments. The first part of cell culture was to test the MitoTracker to see if it would stain the cells. This was done in order to use it as a comparison to the transfection results, to see the localization of the plasmid. These samples will be used as a positive control for the transfection experiments. Three different concentrations were tested, 25 nM, 250 nM, and 500 nM. The best and clearest pictures came from the 500 nM sample. Figure 5 shows the three different samples at a four times zoom. The first picture, letter A, shows the sample at 25 nM. The picture is not as bright and vibrant as the other two and it is not as clear as the other two samples. The middle picture, letter B, shows the sample containing 250 nM of MitoTracker solution. This picture is a lot clearer and more vibrant in most places. However, letter C, is the most vibrant and easy to see. It shows the sample with 500 nM of MitoTracker solution. The whole picture is bright and visible compared to the other two which contain dark spots.

A B C

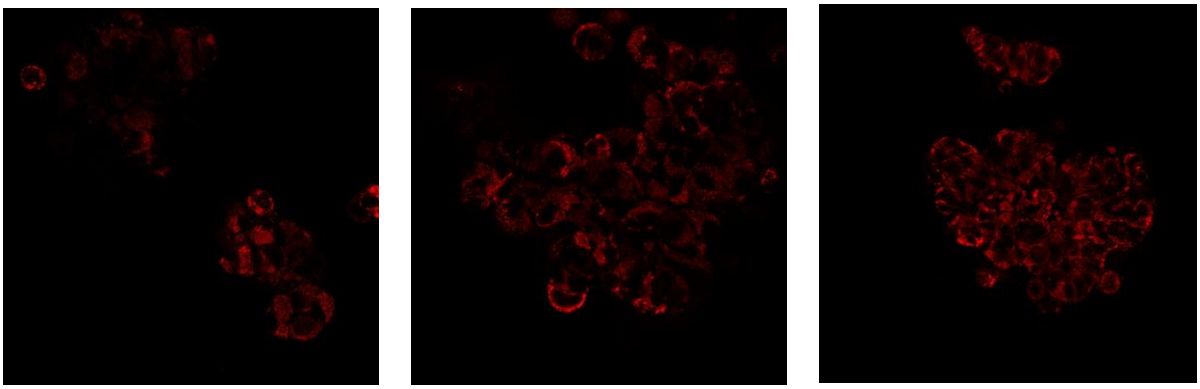

Figure 6. MitoTracker Staining Results.

Following the MitoTracker staining results, the transfection experiments were tested. The purpose of this was to test the efficiency of transfection with the known plasmid and localization points. Two different types of plasmid DNA were used, the first was plasmid DNA with ChR-2, GFP and KCPL and the second was plasmid DNA with ChR-2,GFP and KDEL. KCPL localizes uses to the lysosomes and KDEL localizes to the endoplasmic reticulum. Two different incubation times were tested as well as the two different plasmids. The results below show the transfection results after 24 and 48 hours. Figure 7A shows the transfection experiment with KCPL after 48 hours. Figure 7B shows the transfection with KDEL after 48 hours and figure 7C shows the transfection after 24 hours with KCPL.

A B C

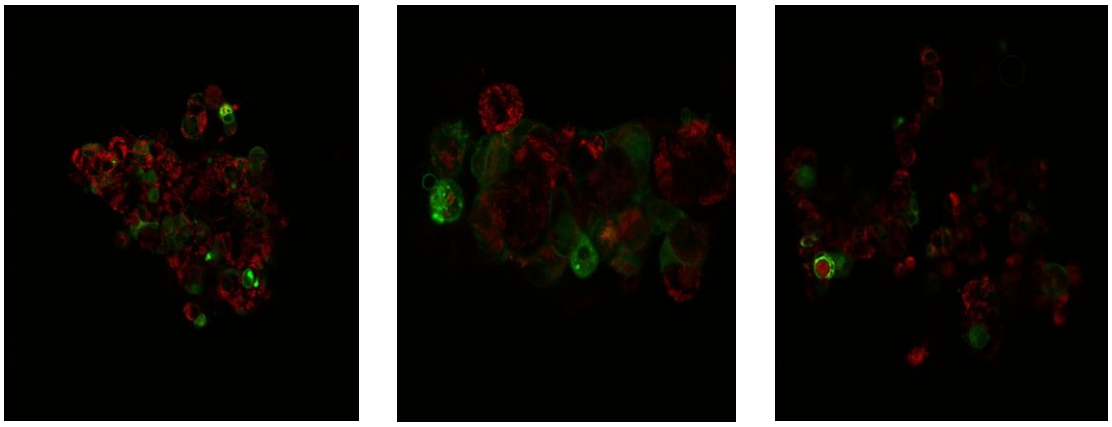

Figure 7. Shows the results of the transfection with KCPL and KDEL

The confocal experiments show that the MitoTracker can successfully stain HEK cells. It also shows that transfection with plasmid DNA is possible. Also the plasmid DNA did localize to the correct location. It is evident from the pictures that the transfection worked because of the localization of the plasmid. As you can see in the picture there are spots where the MitoTracker and GFP overlap creating a yellowish hue. This shows that the two stains are co-localizing in the same location. In the case of the KDEL, it is in the endoplasmic reticulum and for the KCPL, it is in the lysosomes. Because of the yellowish hue, it is evident that the plasmids are in the location that they should be because of the co-localization of stains. Also the pictures show that the best incubation time for transfection is 48 hours. This is because the pictures are clearer and brighter which shows that the GFP had more time to localize and stain the cell in the correct location. The transfection with the fusion protein should localize to the mitochondria which will be the same as the MitoTracker but different from the plasmid DNA.

Future Experiments

This project has the potential to be a great tool for researchers to use toward the study of diseases but there are some experiments that need to be completed before any final conclusions can be drawn as to the exact efficiency of the project.

First of all, the transfection should be tried with the ligation product. By doing this, it will show if it is possible to create an insert that will signal the ChR2 to the mitochondria. This is important because in the future it could be used to insert a different signal sequence leading it to different cells or other locations within the cell.

Second, after the transfection with the ligation product is successful it is important to try the procedure to induce apoptosis. This is important because it will show that the whole project was successful and that it is possible to induce apoptosis using channelrhodopsin-2.

Works Cited

Boyden ES, Zhang F, Bamberg E, Nagel G, Deisseroth K (September 2005). "Millisecond-timescale genetically targeted optical control of neural activity". Nat. Neurosci. 8 (9): 1263–8.doi:10.1038/nn152510.1038/nn1525. PMID 1611644

Dash, Phil. Apoptosis. Reproductive and Cardiovascular Disease Research Group. April 20, 2011 http://www.sgul.ac.uk/depts/immunology/~dash/apoptosis/

Fieldbauer, Katrin, Zimmerman, Dirk, Pintschovius, Verena, Spitz, Julia, Bamann, Christian, Bamberg, Ernst. (July 2009) "Channelrhodopsin-2 is a leaky proton pump" PNAS July 28, 2009 vol. 106 no. 30 12317-12322

Gadsby, David C. (May 2010) "Ion Channels versus ion pumps: the principle difference, in principle" Molecular Cell Biology 2010 May 1 10(5) 344-352

Kim JM, Hwa J, Garriga P, Reeves PJ, RajBhandary UL, Khorana HG (February 2005). "Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops.". Biochemistry 44 (7): 2284–92. doi:10.1021/bi048328i. PMID 15709741.

Nagel, G, Szellas T, Kateriya S, Adeishvili N, Berthold F, Ollig D, Hegemann F, Bamberg, Ernst. (November 2003) "Channelrhodopsin-2, a directly light-gated cation-selective membrane channel" PNAS November 25, 2003 vol. 100 (24):13940-45

Nagel G, Szellas T, Huhn W, Kateriya S, Adeishvili N, Berthold P, Ollig D, Hegemann P, Bamberg E (2003) Channelrhodopsin-2, a directly light-gated cation-selective membrane channel. Proc Natl Acad Sci USA 100 13940–13945

Omura, Tsuneo. (February 1998) "Mitochondria-Targeting Sequence, a Multi-Role Sorting Sequence Recognized at all Steps of Protein Import into Mitochondria." Journal of Biochemistry 123(1010-1016)

Schoenengerger, P, Scharer, Y, Oertner, T. (2010) Channelrhodopsin as a tool to investigate synaptic transmission and plasticity, Exp Physiol June 18, 2010

Zhang, F. et al. Circuit-breakers: optical technologies for probing neural signals and systems. Nature Reviews Neuroscience 8, 577-581 (August 2007)

Zhang, Feng et al. Channelrhodopsin-2 and optical control of excitable cells. Nature Methods 3.10, 785-795 (October 2006)

Zorzos, A. N., Boyden, E. S.*, and Fonstad, C. G. (2010) A Multi-Waveguide Implantable Probe for Light Delivery to Sets of Distributed Brain Targets, Optics Letters 35(24):4133-5.