AN ABSTRACT OF THE THESIS OF

BRYAN GOLDMAN

SUBMITTED ON DECEMBER 15, 2015

TO THE DEPARTMENT OF NEUROSCIENCE

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

OF THE SCHOOL OF SCIENCE AND ENGINEERING

OF TULANE UNIVERSITY

FOR THE DEGREE

OF

MASTER OF NEUROSCIENCE

BY

Bryan Goldman

APPROVED: 

Anne Robinson, Ph.D.
Director

Laura Schrader, Ph.D.

Taby Ahsan, Ph.D.

THE EFFECTS OF OXIDATIVE STRESS ON ADENOSINE RECEPTORS IN SACCHAROMYCES CEREVISIAE

By

Bryan Goldman

A thesis submitted to the faculty of Tulane University in partial fulfillment of the requirements for the degree of Master of Neuroscience

Fall 2015

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ABSTRACT

Oxidative stress is a type of cellular stress that can damage and kill cells. While it is naturally occurring, many non-natural substances found in our environment can also induce the formation of reactive oxygen species (ROS), which then cause oxidative stress within the cell. Oxidative stress has been shown to be involved in the death of neurons in a number of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis. The primary causes for these diseases are still unknown; however, we do know oxidative stress plays a primary role in their development. In conditions where oxidative stress is present, adenosine receptor expression has been upregulated and has played a cytoprotective role, but the specific mechanism of action is unknown.

In this thesis, oxidative stress was studied in a model eukaryote, *Saccharomyces cerevisiae*, and the effects of the expression of the human A_1 and A_{2A} receptors upon stress response was examined. Oxidative stress was induced by the addition of hydrogen peroxide at concentrations of .5 mM, 1 mM, and 2 mM. The growth of cells expressing either A_1-GFP R or A_{2A}-GFP R at the varying hydrogen peroxide concentrations were compared to the parental cells. Confocal microscopy was performed to determine the receptor expression levels, and to confirm the expression of the receptors via their GFP tag. Immunoblots were also performed to assess the receptor expression level at the differing hydrogen peroxide concentrations. A ROS assay was also performed to show

the presence of ROS and oxidative stress in the cells. No significant increase in receptor level expression or localization for either A_1 R or A_{2A} R at the varying hydrogen peroxide concentrations was found. The data did show trends indicating that A_{2A} receptors may help process the oxidative stress better than A_1 receptors and that A_{2A} receptor containing cells had a shorter doubling time. However, more research on this subject should be performed in the future. However, the concentration of hydrogen peroxide should be greatly increased for further experiments in *S. cerevisiae* in order to better differentiate the trends observed.

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Also thank you to my roommates and friends for their support and friendship, specifically Rushil, Justin, and Katie. It has led to many funny conversations about my

precious yeast and my needing to give them loving care at all hours of the day. And if you all didn't keep me distracted when I'm at home, this would have been much more difficult and my life not quite as much fun.

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Chapter 1:

INTRODUCTION

1.1 Neurodegenerative Disease and Oxidative Stress

Neurodegenerative diseases are a class of diseases that primarily affect the neurons in the central nervous system and cause a number of debilitating and generally incurable illnesses. This loss of neurons can present itself as dementia and/or ataxia in humans as seen in Parkinson's disease, Alzheimer's disease, and Amyotrophic Lateral Sclerosis. Although we do not understand all the causes of these diseases, oxidative stress has been indicated in their development and progression (Kim et al., 2007).

Oxidative stress is the cellular stress created by the presence and generation of free radicals, which are molecules with an unpaired electron in their outer orbit, and Reactive Oxygen Species (ROS), which are chemically reactive molecules with an oxygen atom as shown in Image 1.1.1. These species are created through external factors like radiation, organically through cellular metabolism, or through the addition of chemicals like hydrogen peroxide (Uttara et al., 2009). These free radicals can be beneficial in some circumstances, by helping phagocytes destroy engulfed cells and play a role in cell signaling as is the case with nitric oxide (McCord, 2000). However, free radicals can also readily cause oxidative damage to proteins, DNA, and lipids. Damage to these cellular components can lead to cellular death and eventually to the development of neurodegenerative diseases.

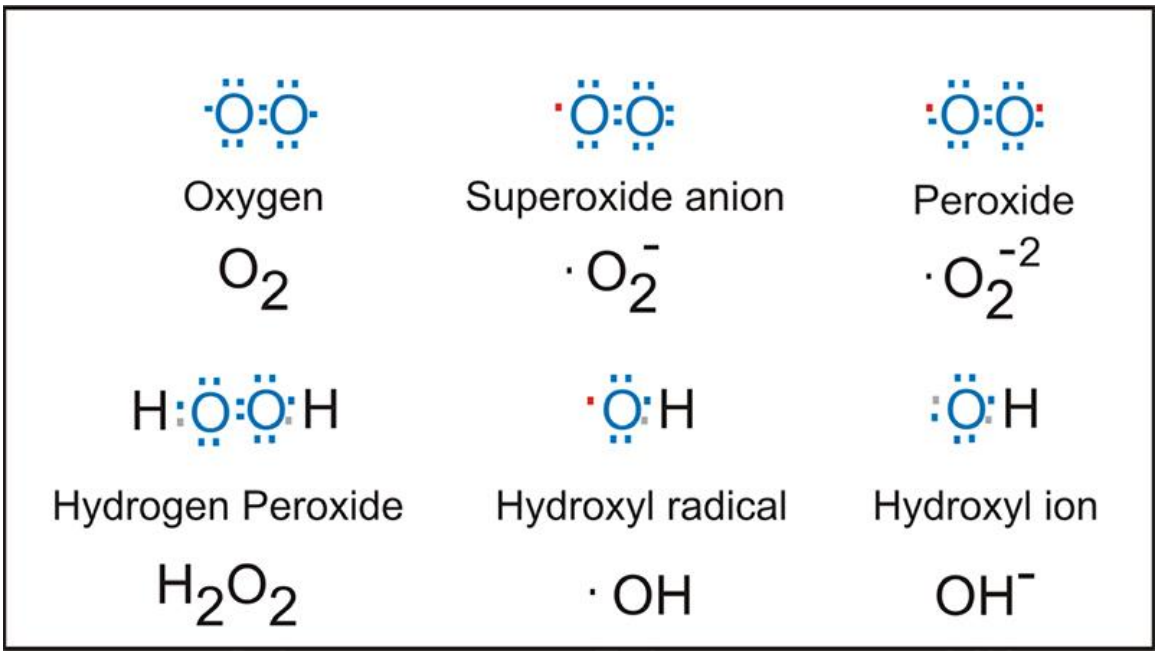

Image 1.1.1 Diagram showing the electron structures of reactive oxygen species. The free radicals are shown in red. It can also be seen how hydrogen peroxide can easily turn into two hydroxyl radicals (Held, 2014).

1.2 Adenosine Receptors, Oxidative Stress, and Neurodegenerative Disease

Adenosine receptors (AR) are a class of G-protein coupled receptors. There are four different adenosine subtypes – A_1, A_{2A}, A_{2B}, and A_3. A_1 R and A_3 R couple with G_i proteins to inhibit adenylyl cyclase and activate phospholipase C. A_{2A} R and A_{2B} R couple with G_s proteins to stimulate the formation of adenylyl cyclase and cAMP (Poulsen et al., 1998). Adenosine receptors are found throughout the body as shown in Image 1.2.1, and are implicated in numerous neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (ALS) (Kim et al., 2007). For example, it has been shown that A_1 R have a high degree of colocalization with the amyloid plaques which are considered a hallmark of Alzheimer's disease. This same study also found that agonists of A_1 R led to an increase in the production of amyloid precursor protein and an increase in tau phosphorylation. The tau phosphorylation may lead to neurofibrillary tangles, another hallmark of Alzheimer's disease (Angulo et al., 2003). An A_{2A} R antagonist (istradefylline) has also been shown to reduce Parkinson's disease symptoms in human patients, demonstrating a potentially integral role of adenosine receptors in Parkinson's disease as well (Jenner et al., 2009). There is also much research that has been performed demonstrating the positive effects of caffeine administration in Parkinson's disease and Alzheimer's disease treatment (Rivera-Oliver & Díaz-Ríos, 2014). Caffeine is a known adenosine receptor antagonist, but also has non-specific effects on a number of other receptors, so distinguishing a direct correlation between caffeine and adenosine receptors is difficult. There is also research showing that in mice used as a model for ALS, A_{2A} R is increased at the onset of symptoms, and A_{2A} R inhibition delays the onset of symptoms. This research also shows

that humans and mouse models demonstrate elevated adenosine levels in cerebral spinal fluid during the onset of ALS symptoms (Ng et al., 2015). In mouse models of Huntington's disease, another neurodegenerative disease, an A_{2A} R antagonist (SCH 58261) modulated the behavioral alterations normally seen in this model (Scattoni et al., 2007). All of these diseases involve both adenosine receptors and oxidative stress, so it is essential we study adenosine receptors and their role in neurodegenerative diseases.

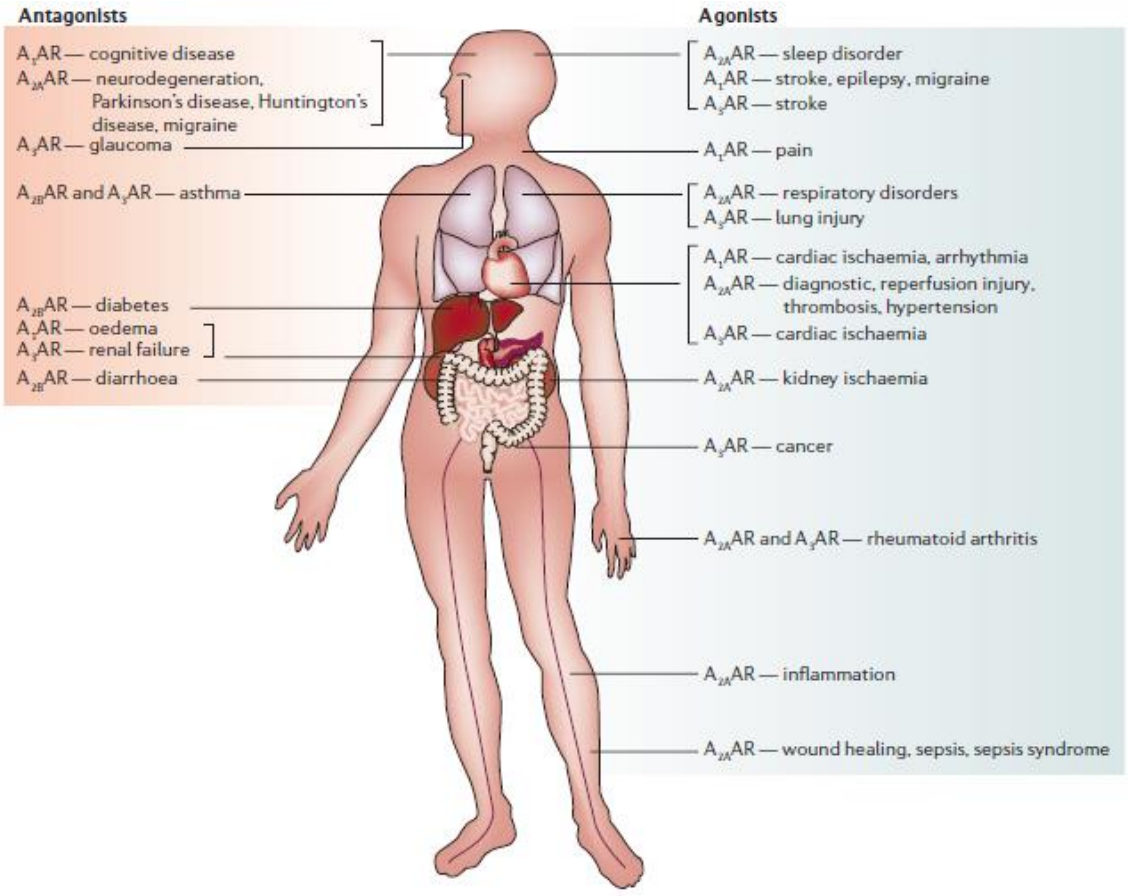

Image 1.2.1 A diagram showing the types of adenosine receptors, and their diverse locations throughout the body (Jacobson et al., 2006).

Due to the associations between neurodegenerative diseases, oxidative stress, and adenosine receptors, research has been performed to examine their relationship. In the presence of ROS causing oxidative stress, A_1 receptors were upregulated in in vitro smooth muscle cells (Nie et al., 1997). It was thought that these A_1 receptors may be providing a cytoprotective role for the cell (Nie et al., 1997, Rudolphi et al., 1992, Yang et al., 2015). A_{2A} receptor agonists have also been shown to reduce oxidative stress in rats, though the role of A_{2A} receptors has not been explored as thoroughly as that of A_1 receptors (Huang, 2003).

Despite the research mentioned above, there has still been a lack of research performed on the direct effects of A_1 and A_{2A} receptors in relation to oxidative stress and neurodegenerative diseases. There have not been comparisons between the two receptor types made, although we do know that on their own they can help a cell survive oxidative stress. Understanding how these receptors handle oxidative stress and which receptor does this better is also integral to our understanding. Learning if the receptors are upregulated, if they are localized differently, if they interact with other receptors, and discovering their intracellular pathways is very important. This thesis will focus on understanding the different effects A_1 and A_{2A} receptors have on cell survivability, and if their receptor expression levels change under oxidative stress. Comparing these to the growth of wild type cells without adenosine receptors will also help us better understand the possible function adenosine receptors play in reducing oxidative stress and potentially increasing cell survivability under these conditions. This of course will teach us more about the role these receptors may play in the development and potential treatment of neurodegenerative diseases.

1.3 Model System

Saccharomyces cerevisiae BJ5464 was used as the model organism for these experiments. It is easily genetically modified, straightforward to grow, and a stable cell line transformed with A_1 R and A_{2A} R was already developed in our laboratory (O'Malley et al., 2009). *S. cerevisiae* is also considered to be a good model for more complex mammalian cells due to similar intracellular pathways (Karathia et al., 2011).

1.4 Project Objective

The objective of this thesis was to determine how the expression of A_1 and A_{2A} receptors in *S. cerevisiae* changed in response to oxidative stress, and whether the expression of the receptors affected cell survivability during oxidative stress induced by hydrogen peroxide. This study was intended to help strengthen the connection between the possible neuroprotective effects of A_1 and A_{2A} receptors in the presence of oxidative stress.

Chapter 2:

MATERIALS AND METHODS

2.1 Transformation

BJ5464 cells transformed with integrating vectors pITy A_1-GFP R or A_{2A}-GFP R to create stable yeast expression cell lines (McCusker, 2008) were used for these studies.

2.2 Media and Growth Conditions

All yeast cells were taken from a -80°C frozen stock, and spread on Agar plates prior to experiments. Agar plates were prepared from an autoclaved solution of 24 g/L Bacto Agar (Research Products International Corporation), 20 g/L Bacto Peptone (Becton, Dickinson and Company), 10 g/L Yeast Extract (Fisher Scientific). 2 g/L glucose was added after autoclaving. The solution was cooled to roughly 50°C prior to pouring, and roughly 20 mL was poured into 10 cm sterile petri plates. BJ5464 yeast containing an integrated gene for A_1 receptor or A_{2A} receptor (McCusker, 2008), were grown on Agar plates containing 200 mg/L of G418 disulfate salt. Wild Type BJ5464 yeast were grown on agar plates lacking G418 disulfate salt. Cells were grown on agar plates at 30°C for 48 to 72 hours.

Yeast cells were then grown in 5 mL of liquid YPD media (10% yeast extract, 20% bacto peptone, and 2% dextrose (Becton, Dickinson and Company)) in autoclaved yeast culture tubes (Fisher Scientific) for 24 hours at 275 rpm and 30°C. After growth in YPD media, the cells were transferred to YPG media (10% yeast extract, 20% bacto

peptone, and 2% galactose (Acros Organics)) in an autoclaved yeast culture tube at 275 rpm and 30°C (Wedekind et al., 2006).

2.3 Dose Response Curves

Following a 12-hour growth of cells on expression media (YPG), one optical density of cells was transferred into a new 5 mL culture tube containing fresh YPG media. After 2 hours of growth at 275 rpm and 30°C, the optical density of the samples were determined, and cultures were then subjected to oxidative stress with hydrogen peroxide at final concentrations of .5 mM, 1 mM, and 2 mM. Hydrogen peroxide concentrations were determined based on previous unpublished research conducted in the Robinson Lab on neuronal SH-SY5Y cells. Control cells lacking hydrogen peroxide treatment also had their OD recorded and remained out of the incubator during this process. All the cells were then grown for an additional 6 hours at 275 rpm and 30°C. The OD was measured and growth was suspended by removing the tubes from the incubator, pelleting cells by centrifugation at 16873 rcf, discarding the supernatant, and freezing the pellets at -20°C. OD was measured using a 1 mL cuvette with 50 μL sample and 950 μL YPG on a NanoDrop 2000c Spectrophotometer (Thermo Scientific).

2.4 Confocal Microscopy Imaging

Confocal microscopy was used to confirm the presence of the adenosine receptors and to determine their relative expression level. A Nikon A1 Plus Confocal Microscope was used. The frozen pellet of yeast cells was suspended in phosphate-buffered saline (Thermo Fisher Scientific), and .2 OD of cells was removed and placed in 1 mL of PBS.

Samples were then washed twice by centrifuging the cells at 1,683 rcf for 1.5 minutes and discarding the supernatant. After the washes 200 μL was added to each well of the room temperature Lab-Tek II Chambered #1.5 German Coverglass System confocal slide (Thermo Fisher Scientific).

Cells expressing A_{2A} R were imaged with transmitted light at a gain of 135, an offset of zero, and 100 X magnification. The GFP tag for A_{2A} was excited at 488 nm, and emission detected at 525 nm with a gain of 70 and power of 1. Cells expressing A_1 R were also imaged with transmitted light at a gain of 135, offset of zero, and 100 X magnification. The GFP tag for A_1 was excited at 488 nm, and emission detected at 525 nm with a gain of 145 and a power of 1. Wild-type cells without adenosine receptors or GFP tags were imaged under these conditions as well.

Four images from each cell type and hydrogen peroxide concentration were captured. These images were taken with the intention of representing a random, but not too crowded section of the slide. The data was analyzed using ImageJ software. Cells were manually traced and compared to blank background of the same area. Six cells were analyzed from each image, so a total of 72 cells were analyzed. The integrated density between the cell measurements and blank measurements was compared to gather the intensity of expression data.

2.5 Reactive Oxygen Species Assay

A reactive oxygen species assay – the "Cellular ROS/Superoxide Detection Assay Kit" (Abcam) – was performed to confirm the presence of oxidative stress within the cells and to examine a possible correlation between hydrogen peroxide concentration and

oxidative stress levels. Cells were collected by centrifugation at 400 x g for 5 minutes at 37°C, and re-suspended at a density of .75 x 10^6 cells per 1 mL PBS (Thermo Fisher Scientific). 100 µL of this resuspension was placed into wells of a 96-well black walled plate (Corning Incorporated). 3 replicates were used for each sample, and 2 measurements per replicate were performed. Cells with no hydrogen peroxide were used for positive and negative controls. The plate was spun at 400 x g for 5 minutes at 30°C and then supernatant was removed. 100 µL of 1X wash buffer provided by the assay was added to each well, then the plate was spun again at 400 x g for 5 minutes at 30°C and supernatant was removed. 100 µL of 1X wash buffer was again added to each well. A reactive oxidative species inducer (pyocanin) and a reactive oxidative species inhibitor (N-acetyl-L-cysteine) was added to cells without hydrogen peroxide to create positive and negative controls as instructed in the assay's protocol. Following a 30 minute dark incubation at 37°C, 100 µL of oxidative stress and superoxide detection reagents were added to the wells also as indicated by the assay's protocol. After the addition of the oxidative stress and superoxide detection reagents, the cells were incubated in the dark at 37°C for 1 hour. After the incubation, products of the reactions were detected on a Synergy H1 microplate reader (Bio-Tek Instruments). For the oxidative stress reagent, a green product is formed and the excitation was 488 nm and emission was detected at 520 nm with a gain of 100. This is sensitive to hydrogen peroxide (H_2O_2), hydroxyl radicals (HO), nitric oxide (NO), and peroxy radical (ROO). It is not as sensitive to superoxide (O_2^-), so the superoxide detection agent is used for superoxide. This was excited at 550 nm and was detected at 620 nm with a gain of 100.

2.6 Immunoblots

Immunoblots were performed to determine relative amounts of adenosine receptors in each cell type. Frozen pellets of cells were lysed using 400 μL of a whole cell lysis buffer solution. The lysis buffer solution was made of 6 mL of Lysis Buffer (10% glycerol, 50 mM NaH_2PO_4, 300 mM NaCl, pH: 8) combined with protease inhibitor and 60 μL of 100 mM PMSF. The pellet was broken up via pipetting with lysis buffer and then roughly 500 μL of .5 mm diameter Zirconia/Silica beads (Thermo Fisher Scientific) were added. The mixture was then vortexed for 1 minute, cooled on ice for 1 minute, in three separate cycles. Then the sample was centrifuged at 16873 rcf for 2 minutes and the supernatant was recovered as the lysed protein.

Following the whole cell lysis, a bicinchoninic acid (BCA) assay (Thermo Fisher Scientific) was performed to determine the exact total protein concentration in each sample in order to standardize the lanes in the following immunoblots. The bichinchoninic acid assay was performed according to a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific). Standards were prepared using bovine serum albumin at 2 mg/mL in 0.9% saline and 0.05% sodium azide (Thermo Fisher Scientific), and a working reagent provided by the kit was added to each sample. Absorbance was then measured in a Synergy H1 Microplate Reader (BioTek) at 562 nm. The standard curve from one of the BCA assay in which protein concentrations for immunoblots was calculated is shown below in Figure 2.6.1.

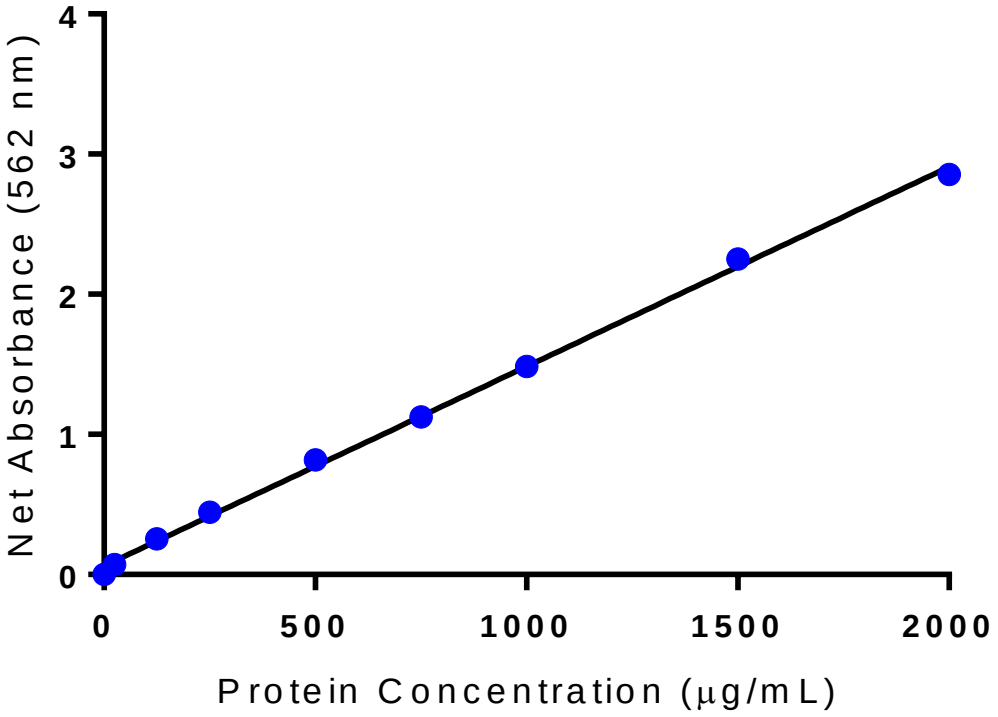

Figure 2.6.1 The standard curve from the BCA assay in which protein concentrations for immunoblots was calculated. This graph has a R^2 value of .9983.

20 μg of protein were loaded into each well of a 12 well 12% Tris-glycine gel (Thermo Fisher Scientific). 3 μL of a Precision Plus Protein Standard (Bio-Rad Laboratories) was loaded into a single well on each gel. The gel was electrophoresed for 70 minutes at 400 mA and 125 V. For Western analysis, a complete protein transfer onto a .2 μm nitrocellulose membrane using Trans-Blot Turbo Transfer System (Bio-Rad Laboratories) was performed. Then the membrane was placed in 25 mL of 5% milk (Research Products International Corporation) in Tris-buffered saline (Bio-Rad Laboratories) and 1% Tween (Fisher Scientific) overnight while rocking at 4°C. After the overnight blocking an anti-GFP polyclonal primary antibody (Abcam ab6556) was added in a 1:3000 ratio in TBST. This was incubated while rocking at room temperature for 2 hours. Then the primary antibody was removed and the membrane was washed three times with 25 mL TBST for five minutes per wash. Then an anti-rabbit secondary antibody (Abcam ab16284) was added in a 1:3000 ratio in TBST. This was also incubated while rocking at room temperature for 1 hour. The membrane was again washed three times with 25 mL TBST for five minutes per wash. Then detection was performed with Luminata Forte Western HRP Substrate according to the protocol and imaged with a UVP BioSpectrum 610 Imaging System (UVP).

We are unable to compare A_1 R and A_{2A} R directly because the inherently stronger expression of the GFP tag in A_{2A} R. This is reflected in the exposure time required for each receptor. A_1 R required a 3 minute exposure to obtain an image similar to A_{2A} R which only required a 30 second exposure, while both immunoblots were carried out with identical procedures and amounts of protein.

Chapter 3:

RESULTS

The objective for this thesis work is to determine if the expression of A_1 and A_{2A} receptors in *S. cerevisiae* changes during oxidative stress induced by treatment with hydrogen peroxide. These results will help strengthen the connection between the possible neuroprotective effects of A_1 and A_{2A} receptors in the presence of oxidative stress, which has been implicated in a number of neurodegenerative diseases.

3.1 Dose Response Curves

Dose response curves were performed to see how the optical density of WT, A_1-GFP receptor and A_{2A}-GFP receptor expressing cells grew in the presence of varying concentrations of hydrogen peroxide, where growth was used as a proxy for overall cell health. Optical densities were recorded at 2 hours and 8 hours of growth after seeding at 1 OD in YPG. Each cell type was treated with 0.5 mM, 1 mM, or 2 mM hydrogen peroxide, with 0 mM of hydrogen peroxide as a control. Independent growth studies were performed at least 4 times for each cell type. Figure 3.1.1 shows the average change in OD over the 6 hour growth window. Despite large error bars, a general trend is clearly observed – higher concentrations of hydrogen peroxide lead to smaller differences in OD over the growth period. This means that at higher hydrogen peroxide concentrations, the cells are not growing as well as they would in normal circumstances, which is to be expected. We do also see a significant difference in the growth of both

WT and A_1 R expressing cells after the addition of hydrogen peroxide. However, we do not see any significant differences in A_{2A} R expressing cells with the addition of hydrogen peroxide.

Figure 3.1.2 shows the average doubling time based on change in OD over the 6 hour growth period for each cell type and hydrogen peroxide concentration. A Two-way ANOVA shows that there is a significant difference in doubling times between WT and A_{2A} R cells ($p < .0001$), and between A_1 R and A_{2A} R cells ($p < .01$). The data shows that at 2 mM, A_{2A} R cells grew at 3 times the rate of WT cells and 2.5 times the rate of A_1 R cells. This is evidence that the A_{2A} R cells are able to handle the addition of hydrogen peroxide and oxidative stress better than WT and A_1 R expressing cells. It also shows that the major effects are not seen until at least 2 mM of hydrogen peroxide has been added.

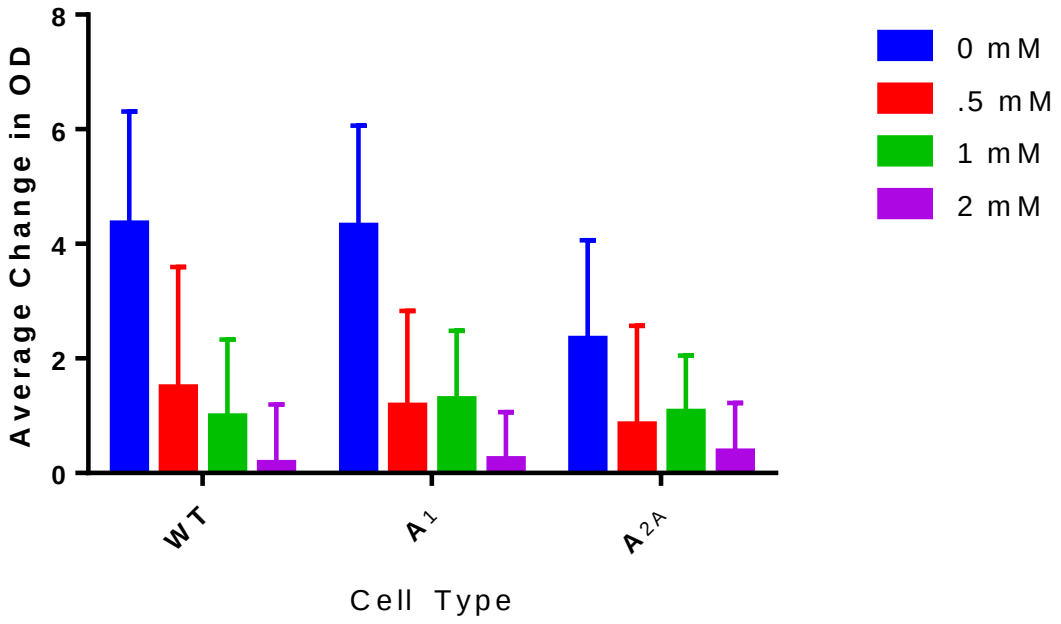

Figure 3.1.1 The average change in optical density over a 6 hour growth period. Cells were seeded at 1 OD and measurements were compared at 2 and 8 hours post seeding. A Two-way ANOVA test with 95% confidence interval showed that there are significant differences between all cells with hydrogen peroxide and those without hydrogen peroxide in WT and A_1 R containing cells, but not A_{2A} R cells. This shows that the addition of hydrogen peroxide does significantly impact the growth of cells and implies that the cells with A_{2A} R may handle the stress caused by the addition of hydrogen peroxide better than WT and A_1 R cells.

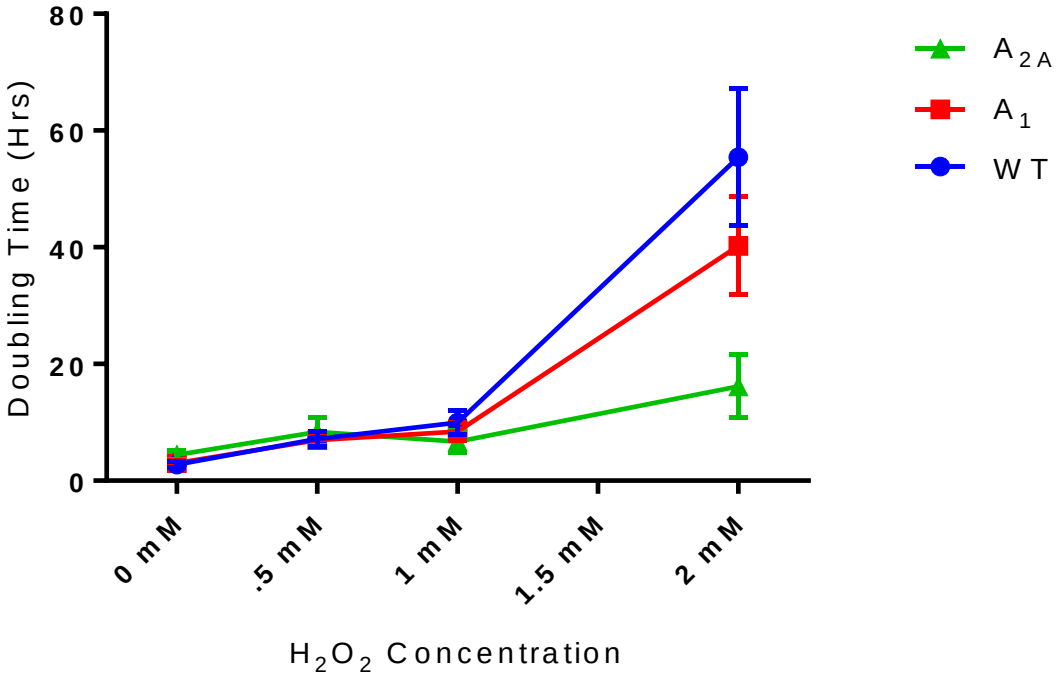

Figure 3.1.2 The doubling time in hours as measured during the 6 hour growth period. Through a Two-way ANOVA test it is shown that the doubling times are significantly different between WT and A_{2A} R cells ($p < .0001$) and A_1 and A_{2A} R ($p < .01$) cells at a concentration of 2 mM hydrogen peroxide. This graph shows that A_{2A} R cells were able to grow at a much higher rate compared to A_1 and WT cells at 2 mM. It is also noteworthy that at 0 mM, .5 mM, and 1 mM there was no significant differences in doubling times between the groups.

3.2 Confocal Microscopy

Confocal microscopy was used to verify that the cells containing A_1 R and A_{2A} R were expressing those receptors, and to establish whether there was a change in receptor expression with increasing hydrogen peroxide concentration.

Cells were imaged from previously frozen samples taken at 8 hours of growth in new media. Images were captured in the manner described in chapter 2.4, with each image intended to represent a random, but not too crowded area of the slide. The images were analyzed using ImageJ software. Cells were manually traced and were compared to a non-fluorescing background of the same area, and integrated density was recorded. The average intensity for these cells is shown in Figure 3.2.4 below. The data does not appear to show any significant trend with hydrogen peroxide treatment. It does demonstrate that the fluorescence from the A_{2A} R with a GFP tag is much greater than A_1 R with a GFP tag. This was also reflected in the gain used to detect images, as a gain of 135 was used for A_{2A} R and a gain of 70 was used for A_1 R. This difference in intensity was anticipated, as previous studies show that A_{2A} R with a GFP tag has improved expression, and thus increased intensity (O'Malley et al., 2007). Wild-type cells have a very low fluorescence, once again confirming the fact that the GFP tag is functional in the A_1 R and A_{2A} R cells. Background fluorescence in WT cells results from a combination of residual media affects and some naturally fluorescent host proteins.

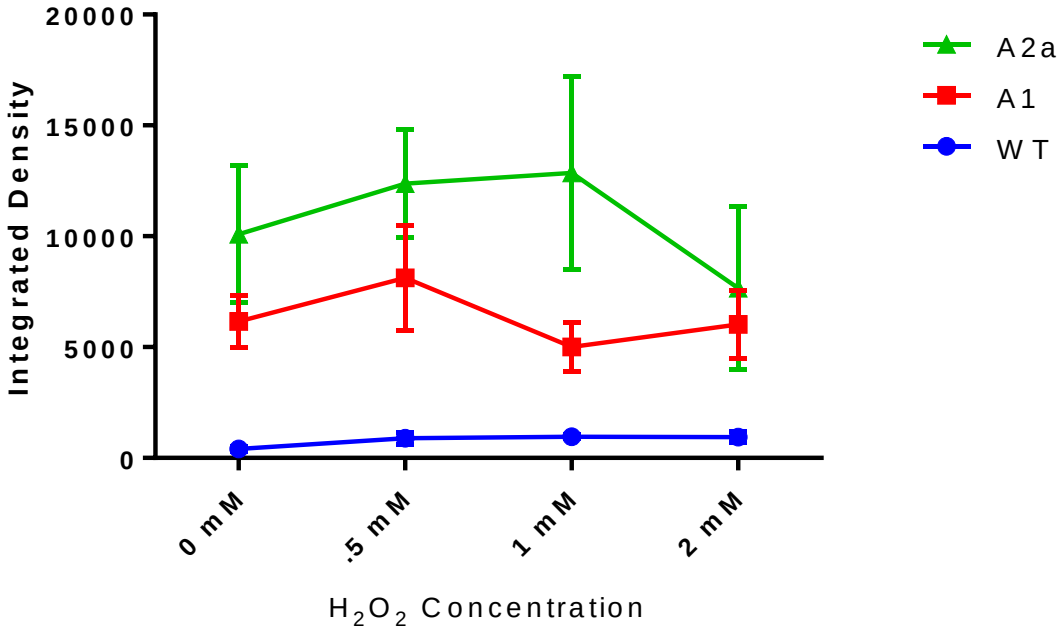

Figure 3.2.1 The integrated density for WT, A_1 R, and A_{2A} R were measured and compared. Nothing significant is recorded other than that A_{2A} R and A_1 R fluoresce confirming the presence of the GFP tag on the receptors, and that WT has a very low baseline fluorescence.

Representative images taken with both transmitted light and with a FITC light at 488 nm. Below are images of A_{2A} R expressing cells with an overlay of both transmitted and FITC (488 nm) light (Image 3.2.1), and then transmitted (Image 3.2.2) and FITC (488 nm) light (Image 3.2.3). There are also images of an A_1 R overlay (Image 3.2.4) and a WT overlay (Image 3.2.5) for comparison.

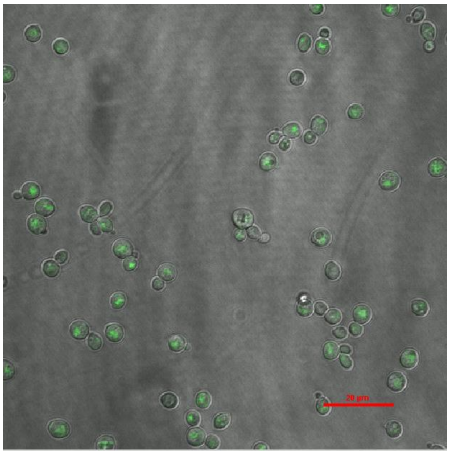

Image 3.2.1 A_{2A} R cells with .5 mM hydrogen peroxide imaged at 100 X with an overlay of both FITC (488 nm) and transmitted light.

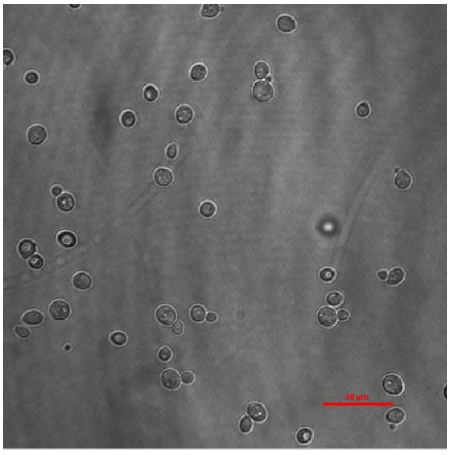

Image 3.2.2 A_{2A} R cells with 0 mM hydrogen peroxide imaged at 100 X with transmitted light.

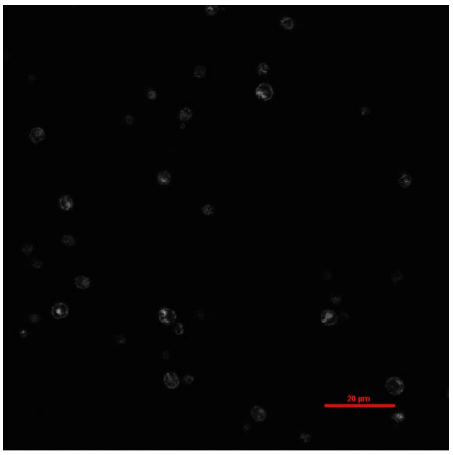

Image 3.2.3 A_{2A} R cells with 0 mM hydrogen peroxide imaged at 100 X with FITC light (488 nm). This is identical to Image 3.2, but with FITC light instead of transmitted light.

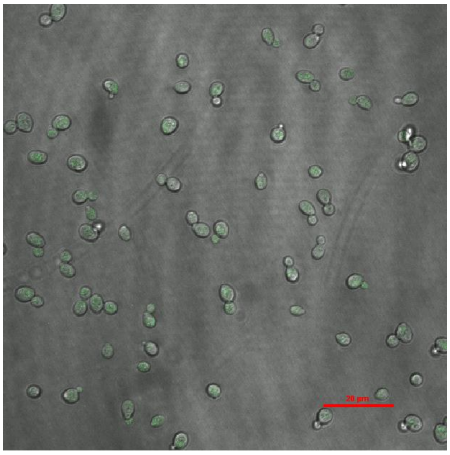

Image 3.2.4 A_1 R cells with 2 mM hydrogen peroxide imaged at 100 X with an overlay of both FITC (488 nm) and transmitted light.

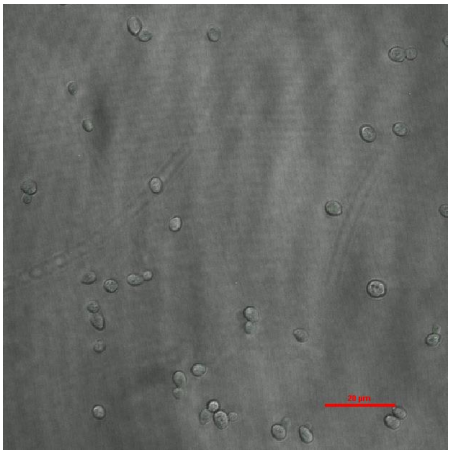

Image 3.2.5 WT cells with 1 mM hydrogen peroxide imaged at 100 X with an overlay of both FITC (488 nm) and transmitted light.

3.3 Reactive Oxygen Species Assay

The reactive oxygen species assay provided through an Abcam kit was used to confirm and analyze the presence of oxidative stress in the different cell types and to determine if there was a correlation between reactive oxygen species levels and hydrogen peroxide concentrations in the cells.

The assay confirmed the presence of reactive oxygen species in all the cells at all four concentrations of hydrogen peroxide. Surprisingly, under these conditions, there are reactive oxygen species in untreated cells, indicating some level of background stress under our culture conditions. In Figure 3.3.1 the data from one of the assays is shown. A Two-way ANOVA test with a 95% confidence interval showed that the negative control is significantly different than all the cells with hydrogen peroxide. This indicates that there is significant ROS in all of the cells with hydrogen peroxide added. There is also a significant difference between A_1 R and A_{2A} R expressing cells with 0 mM and 1 mM hydrogen peroxide, indicating that the addition of hydrogen peroxide does create ROS in these cells.

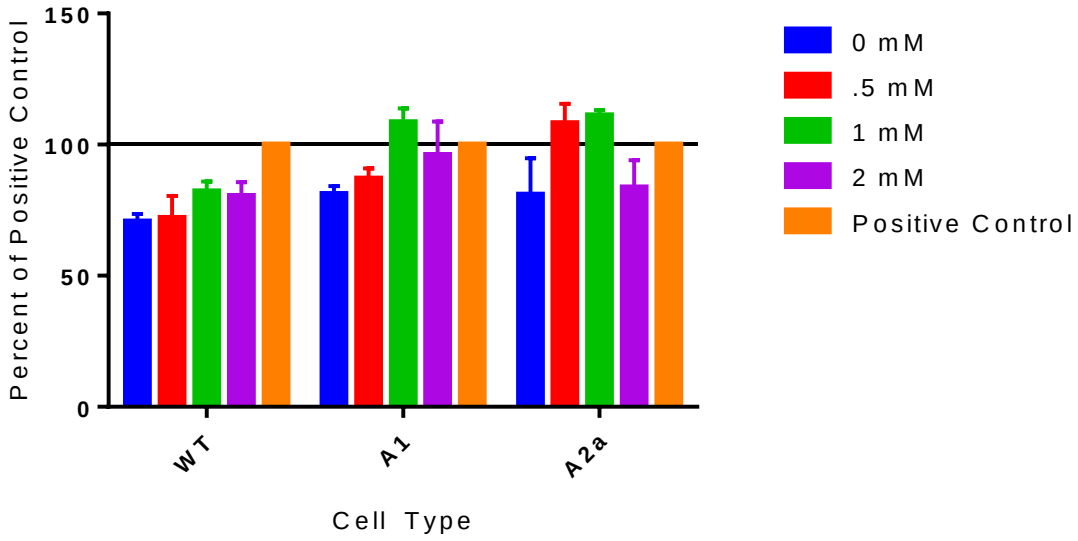

Figure 3.3.1 The ROS standardized to the positive controls as measured by the "Cellular ROS/Superoxide Detection Assay Kit" (Abcam). A Two-way ANOVA test with a 95% confidence interval showed that there are significant differences between the negative controls (not shown) and the cells with .5 mM of hydrogen peroxide added. This establishes the fact that there is significant ROS in the cells with .5 mM hydrogen peroxide added. There is also significant differences between 0 mM and 1 mM concentrations in both A_1 R and A_{2A} R expressing cells, indicating that the addition of hydrogen peroxide creates significant ROS in these cells.

3.4 Immunoblots

Cells were lysed and western blotting was performed as described in chapter 2.6 to measure levels of A_1 and A_{2A} receptor expression at varying concentrations of hydrogen peroxide and oxidative stress. A GFP antibody was used to identify A_1 R (36 kDa) and A_{2A} R (40 kDa). Relative density was calculated using ImageJ software. Figure 3.4.1 shows the relative density of GFP tagged A_1 and A_{2A} receptors at .5 mM, 1 mM and 2 mM hydrogen peroxide compared to those in cells without any hydrogen peroxide added. There does not appear to be any significant trend in decreasing or increasing expression of a receptor at higher hydrogen peroxide concentrations. This agrees well with the data obtained from the confocal images.

Image 3.4.1 demonstrates a representation of the immunoblot used for analysis.

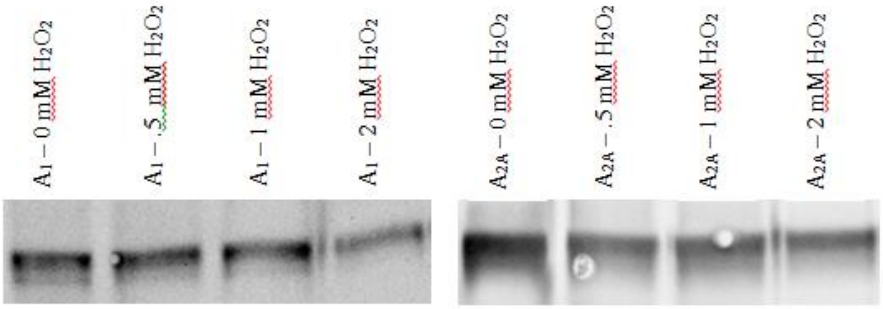

Image 3.4.1 Representative immunoblots of both A_1 R and A_{2A} R. These were imaged on different membranes due to the higher expression of A_{2A} R.

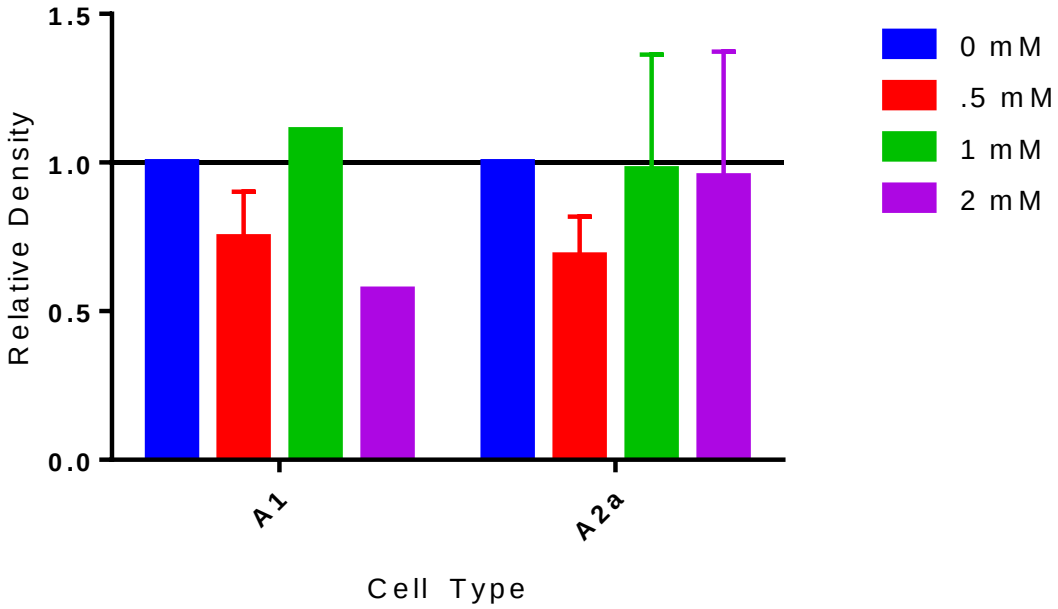

Figure 3.4.1 The relative density of cells with hydrogen peroxide standardized to the cells without hydrogen peroxide. Cells were lysed using bead beating and lysis buffer solution. A BCA analysis was performed to calculate the exact amount of protein per sample. 20 µg of protein was loaded into each well, and was run on a Tris-glycine gel for 70 minutes at 400 mA and 125 V as described in Chapter 2.6. Protein was transferred to a nitrocellulose membrane and blocked for nonspecific binding with milk overnight. It was incubated with anti-GFP polyclonal primary antibody for 2 hours then an anti-rabbit secondary antibody. The blot was imaged with a UVP system and analyzed with ImageJ software.

Chapter 4:

SUMMARY AND FUTURE DIRECTIONS

4.1 Summary

This work focused on the relationship between two types of adenosine receptors, A_1 and A_{2A}, and oxidative stress. Oxidative stress has been observed in Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (Robberecht, 2000; Zhou et al., 2008; Pimental et al., 2012). The A_1 receptor is located in the brain, is upregulated during oxidative stress, and is cytoprotective for the cell (Nie et al., 1998, Rudolphi et al,. 1992). The A_{2A} receptor is another adenosine receptor also located in the brain and has been shown to reduce oxidative stress (Huang, 2003). Establishing whether or not there is a direct correlation between oxidative stress and the expression of A_1 and A_{2A} receptors, and determining if the A_1 or A_{2A} receptors help limit oxidative stress and increase cell survivability could have important implications in research focusing on neurodegenerative diseases implicated with oxidative stress.

The results described here do appear to establish a definitive difference with the growth of A_{2A} R cells compared to A_1 R and WT cells at 2 mM of hydrogen peroxide. This is seen in Figure 3.1.2 where the average doubling time is shown. This is evidence that the A_{2A} receptor handles oxidative stress better than A_1 receptors. However, that data only demonstrates significance at 2 mM, and 6 hours after the addition of hydrogen peroxide. A very different trend may be seen at different time points or higher hydrogen peroxide concentrations.

An interesting trend is also shown in Figure 4.1.1 below. Here the growth of the cells is shown relative to the 0 mM condition. At low concentrations of hydrogen peroxide (0 mM, .5 mM), WT cells grow better than adenosine expressing cells. While at 2 mM hydrogen peroxide, A_{2A} R expressing cells have increased growth compared to A_1 R expressing cells and WT cells. This "adaptive swing" with higher concentrations of hydrogen peroxide is very intriguing since it seems to provide more evidence that the response of A_{2A} R cells is different than A_1 R and WT. Knowing why this adaptive swing occurs could be very important to our overall understanding of adenosine receptors and their cytoprotective role. It is important to continue research to confirm this behavior, and to determine why A_{2A} R might play this role.

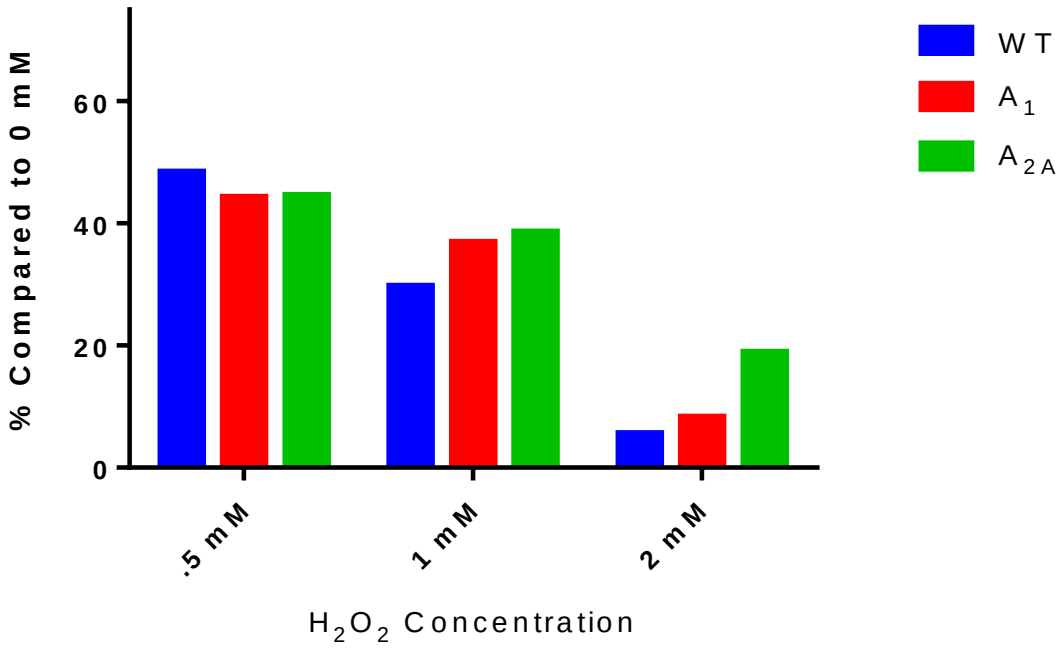

Figure 4.1.1 The growth of cells with hydrogen peroxide relative to the growth of cells without hydrogen peroxide is shown. The trend shown here suggests that there is reduced growth with increased hydrogen peroxide concentrations. However, A_{2A} R containing yeast show improved growth at higher hydrogen peroxide concentrations compared to A_1 R and WT cells.

There does not appear to be an upregulation of either A_1 R or A_{2A} R under oxidatively stressed conditions, as shown by confocal and immunoblot data, but again this does not definitively mean that there is not an upregulation.

4.2 Future Work

There were some trends noticed in this data that imply a connection may exist as well as other research performed in other cell types (Nie et al., 1998, Gołembiowska et al., 2012, Kalkan et al., 2009). Continuing analysis of the possible link between adenosine receptor expression and oxidative stress is necessary, as well as continued work to determine if either A_1 R or A_{2A} R provides more protection against oxidative stress for the cell.

The future studies, if carried out in yeast, will need to be performed with greatly increased concentrations of hydrogen peroxide to induce significantly more ROS and oxidative stress. This became evident since identifying significant differences between hydrogen peroxide concentrations was difficult. This is despite the fact that much research involving *S. cerevisiae* and ROS used hydrogen peroxide concentrations similar to ours (Izawa et al., 1995; Spencer et al., 2014).

As well as increasing hydrogen peroxide concentrations, additional experiments should be performed to determine if differences in receptor localization exist during increasing oxidative stress for either A_1 R or A_{2A} R. It will also be important to determine if the potential changes in receptor expression or cell survivability are due to a temporal effect. For example, the "adaptive swing" seen in Figure 4.1.1 may not be observed at 8 hours after the addition of hydrogen peroxide, even though it is observed at

6 hours. There may also be an increase in receptor expression early on that helps cell survivability later, when we may not see evidence of this upregulation. This could help increase our understanding about what may be activating the receptors and how exactly they work.

Understanding if changes other than an upregulation of the receptors occurs under increasing oxidative stress levels may help us realize the mechanism behind the protective role of adenosine receptors. Research has shown that A_{2A} R may prevent oxidative stress related cell death through Protein kinase A (PKA) dependent pathways, and that it may regulate mitochondrial ATP channel activity (Huang, 2003). PKA is activated relatively early on in most intracellular pathways, so studying the downstream effects after adenosine receptor expression may illuminate other stages in the complex intracellular pathways. These of course help us better understand how adenosine receptors work and may provide drug targets in the future.

Valuable research can also be performed in more complex neuronal cells. These would require lower hydrogen peroxide concentrations to induce significant oxidative stress, and would be better models to understand the pathways in which adenosine receptors may provide a protective role in neurodegenerative diseases. After the pathways are understood, targets may be identified and testing may be possible in transgenic mice where these pathways are altered.

It is also valuable to explore the potential role A_{2B} and A_3 receptors may play in oxidative stress management, even though A_{2B} R is only poorly expressed in the brain. A_{2B} R has been shown to modulate the function of A_1 R in mice (Goncalves et al., 2015), so its role may be larger than initially predicted based off its expression.

The study of the interaction between adenosine receptors and oxidative stress can help us better understand how neurodegenerative diseases like Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis function, and grow to understand how adenosine receptor activation may help prevent the onset or progression of these diseases.

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