Studying whether C-C Motif Chemokine Ligand 11 (CCL11) Induces Reactive Oxygen Species in Microglial Brain Macrophages

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Abstract

Neurodegenerative disease is a process in which cells within the nervous system are damaged or die due to conditions in the brain that influence their well-being. Elevated levels of CCL11, an age-related chemokine, have been linked to neurodegenerative disease. Along with CCL11, there is also the increased observance of excess reactive oxygen species (ROS); which are free radicals that damage cellular DNA, RNA, and proteins, leading to the death of cells. This study investigates the impact that CCL11 has in the production of ROS in brain macrophages, known as microglia. Along this line, we hypothesize that CCL11 would activate microglia and increase extracellular ROS in the brain. Leading to the damage of the neuronal tissues and the development of neurodegenerative diseases, such as dementia. To study this hypothesis, we used in vitro cell culture techniques with the microglial cell line, SIM-A9. Results indicate a significant increase in the production of both intracellular and extracellular H_2O_2, the primary ROS investigated. Furthermore, a potential underlying mechanism that may regulate the production of ROS by CCL11 in microglia was proposed. Understanding the mechanisms that underlie CCL11-mediated ROS production in microglial cells, may provide valuable insight into the pathogenesis of many neurodegenerative disorders. Leading to the development and use of potential therapeutic strategies.

Introduction

Reactive oxygen species (ROS) are natural byproducts of cellular metabolism; however, in excess, they cause damage to cellular DNA, RNA, lipids, and proteins. Elevated levels of ROS have been viewed in many different human diseases, including those that affect the brain: Alzheimer's Disease, Parkinson's Disease, and Huntington's Disease. Oxidative stress due to excess ROS has been linked as a major mechanism of neurocognitive disease advancement (Manoharan al., 2016). A cellular culprit for the production of ROS within the brain is microglial cells, which are responsible for the development and maintenance of neurons. The stimulation of microglial cells via the chemokine CCL11 (Also known as eotaxin-1) which binds to the microglial CCR3 receptor, upregulates the production of ROS within the brain. Recent studies have made ties to CCL11 being a specific biomarker that may be indicative of neurocognitive disease presence and progression. It is also suggested that the serum level of CCL11 protein is increased by aging in humans.

In the research study conducted by Parajuli et al. (2015), it was found that astrocytes within the brain are primarily responsible for the release of CCL11 in the brain and lead to elevated levels of CCL11 in the cerebrospinal fluids. Whereas, microglia express the CCL11 receptor (CCR3) which upregulates NOX-1 (NADP-oxidase 1) leading to the greater conversion of oxygen to superoxide species. Therefore, it promotes excitotoxic neuronal death within the brain due to oxidative stress caused by ROS (Parajuli et al., 2015). The study was able to make numerous conclusions regarding the effects of CCL11 on pathways within the brain; however, the study was based on cell cultures with glutamate to induce glutamate-mediated neuronal cell death. Accordingly, the harmful effect of CCL11 on the brain remains elusive.

As discussed by Sirivichayakul et al. (2019) in the introduction to their study, peripheral CCL11 is also transported through the blood-brain barrier and accumulates in many brain regions. It also refers to the idea that "eotaxin could be an independent predictor of cognitive impairment in older individuals" (Sirivichayakul et al., 2019). Although this study makes no mention of ROS in its findings, it does heavily discuss mental and cognitive symptoms that may arise from individuals with elevated CCL11 levels, making further Research into this chemokine scientifically significant for the aging population. With the ability of the CCL11 chemokine to cross the blood-brain barrier it draws to question the effects of peripheral CCL11 on microglial cells and the subsequent production of ROS within the brain.

Rationale

Although CCL11 does serve a vital role in the immune system and the inflammatory response, the natural increase of this chemokine with age and the following reduced neurogenesis draws to question the involvement in mood and cognitive dysfunction (Ivanovska et al., 2020). With microglial cells being the significant linkage between CCL11 and the production of ROS, further research into CCL11, its binding to microglial cells, and the quantities at which reactive oxygen species are produced are needed. Currently, the effect of CCL11 on brain macrophages has yet to be uncovered, and the mechanism by which CCL11 activates target cells is unknown.

According to McDonald (2017), the increase in the prevalence of neurodegenerative disease doubles every 20 years with the predicted incidence of dementia being 115 million people worldwide by 2050. Although there are likely many factors that play into the progression

of neurodegenerative disease, the induction of microglia ROS production by the stimulation of CCL11 has yet to be fully uncovered. Through this research, the influence of CCL11 stimulation on in vitro SIM-A9 (Microglial cells) will be used to produce quantitative results that are representative of the amount at which these cells produce ROS. Along this line, our research results can provide important insight into explaining the mechanism of neurodegenerative disease development.

Methodology and Materials

Overview

To test the hypothesis that CCL11 stimulates microglial cells to produce ROS in the brain, we will be treating SIM-A9 cell cultures with various levels of CCL11 for 8 hours. SIM-A9 is a microglial cell line derived from the cerebral cortexes of mouse pups and provides an effective way to study microglial cells in vitro. SIM-A9 will be plated and allowed to proliferate until the density of cells is high, with ~80% plate coverage. After a media change with DMSO (10% Fetal Bovine Serum, 1% Penicillin Streptomycin), the cells will then be treated with CCL11 at a quantity ranging from 12.5-200 ng/mL, and then placed to incubate for 8 hours along with untreated controls. Using CCL11 at this range of doses will allow for the determination of an efficacious dose as well as give insight into the mechanism by which CCL11 activates microglial cells. Once the 8-hour incubation period has been completed, the cell lysates and cell supernatant will be evaluated for ROS production through the use of the ROS-GLO™ H_2O_2 Assay by Promega and an illuminator that measures the fluorescence of each sample.

Cell Culture

Using homogeneous DMSO medium treated with 10% fetal bovine serum, and 1% Penicillin-Streptomycin, stock Sim-A9 cells were cultured in sterile petri dishes and allowed to proliferate until their confluency was approximately 80%. During the duration of this growth period, the cells were incubated at 37*C with 5% CO_2 , while their growth was monitored under a microscope, with routine medium changes every 2-3 days depending on the coloration of the media. Sterile conditions were maintained and practiced for the entirety of the growth phase, and no notable signs of contamination were observed in the cultures used in this experiment.

To investigate the underlying cellular and molecular mechanisms behind microglial cells' interactions with CCL11, Petri dishes 1 - 6 of SIM-A9 cells with confluency of ~80% were treated with the following CCL11 quantities respectively: 0 ng/mL (Control), 12.5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL. These cultures were then allowed to incubate at 37*C with 5% CO_2 for exactly 8 hours. After this period, the cell lysates and supernates (cell medium) were harvested and placed in the -80*C freezer for storage until the testing of Reactive Oxygen Species could be performed. Cell lysates were prepared in a RIPA (radioimmunoprecipitation assay) buffer, which allows for the rapid lysis and extraction of cellular contents, prior to freezing.

ROS-GLO™ H_2O_2 Assay

Through the use of the ROS-GLO™ H_2O_2 Assay, luminescence from the presence of hydrogen peroxide in each sample was obtained through the use of a luminator and recorded as

Relative Luminescence Units. For the completion of this experiment, we tested the supernatants of each petri dish six times throughout three trials (2 samples each trial), and the cell lysates twice on the first day of testing. Cell lysates were not the primary focus of this study due to the main hypothesis being focused on the possible damage to surrounding neuronal tissues. Cell lysates were tested on the first day to establish a baseline for the SIM-A9 cell cultures. With the time-sensitive nature of this experiment, there were a total of 12-18 samples per testing day, allowing adequate time for the preparation and testing of each. All samples were taken from the -80*C freezer and placed on ice to warm at a steady pace. Contents of the ROS-GLO™ H_2O_2 Assay were also placed on ice to thaw.

To form the H_2O_2 substrate solution, we combined 5µL of H_2O_2 substrate and 400 µL of dilution buffer in an Eppendorf tube and stored it on ice. After adding 60 µL of each resuspended sample into marked Eppendorf tubes, we then added 15 µL of the substrate solution into each testing sample. Each Eppendorf now had a sample volume of 75 µL, and lights were turned off for the remainder of the experiment. We then formed the ROS-GLO™ Detection Solution by combining 2.5 mL of luciferin detection agent, 25 µL of D-cystine, and 25 µL of signal enhancer, then vortexed to make a homogenous mixture. Then, 75 µL of the detection solution was added to each Eppendorf tube containing the 75 µL of the sample, with a 60-second interval between each addition to allow for the testing of samples after the 20-minute wait between addition and testing.

After the 20-minute wait time from the first addition of the detection solution, we processed each sample through a luminator and recorded the maximum fluorescence of each sample for 30 seconds, and allocated 30 seconds for recording and the sample changing

process (60 seconds total between tested samples). Through the use of this detection method, the H_2O_2 substrate reacts with the presence of H_2O_2 to produce the luciferin precursor. Upon addition of the ROS-GLO™ Detection Solution, the precursor is converted to luciferin which reacts with the recombinant luciferase to generate a luminescent signal proportional to the H_2O_2 concentration (Duellman, 2013). A luminator is used to quantify the amount of light emitted from each sample in relative light units per second (RLU/s).

Data Analysis

Upon the collection of data, RLU/s values obtained from the testing of samples were imputed into an Excel File and plotted against their corresponding CCL11 treatment value. A linear trendline was then computed by the Excel software as well as an R^2 value to be used in the statistical analysis of the collected data. All samples used were standardized among the completed trials.

Results

RLU values from trail 1:

Name of Sample	Cell Supernatant (RLU/s)	Cell Lysate (RLU/s)
SIM-A9- 0 ng/mL CCL11 - Sample 1 (Petri dish 1)	96,345	206,739
SIM-A9- 0 ng/mL CCL11- Sample 2 (Petri dish 1)	108,457	299,000
SIM-A9-12.5 ng/mL CCL11 - Sample 1 (Petri Dish 2)	72,000	252,672
SIM-A9-12.5 ng/mL CCL11- Sample 2 (Petri Dish 2)	65,000	198,203
SIM-A9-25 ng/mL CCL11 - Sample 1 (Petri Dish 3)	107,024	199,620
SIM-A9-25 ng/mL CCL11 - Sample 2 (Petri Dish 3)	145,550	287,873
SIM-A9- 50 ng/mL CCL11 - Sample 1 (Petri Dish 4)	178,411	360,004
SIM-A9- 50 ng/mL CCL11 - Sample 2 (Petri Dish 4)	126,043	299,870
SIM-A9-100 ng/mL CCL11- Sample 1 (Petri Dish 5)	172,000	257,000
SIM-A9 - 100 ng/mL CCL11- Sample 2 (Petri Dish 5)	109,030	297,742
SIM-A9 - 200 ng/mL CCL11 - Sample 1 (Petri Dish 6)	133,478	351,420
SIM-A9- 200 ng/mL CCL11 - Sample 2 (Petri Dish 6)	120,746	246,000

Figure 2A: *Maximum RLU Values from SIM-A9 Microglial Cells Stimulated by CCL11 during day 1 of testing. A notable difference in RLU/s values between supernatant and cell lysate. Cell Lysate has higher quantities of H_2O_2 representing intracellular ROS production under normal and stimulated conditions.*

RLU values from trial 2 of only Supernatant:

Name of Sample	Cell Supernatant (RLU/s)
SIM-A9- 0 ng/mL CCL11 - Sample 3 (Petri dish 1)	65,556
SIM-A9- 0 ng/mL CCL11- Sample 4 (Petri dish 1)	77,880
SIM-A9-12.5 ng/mL CCL11 - Sample 3 (Petri Dish 2)	83,880
SIM-A9-12.5 ng/mL CCL11- Sample 4 (Petri Dish 2)	98,750
SIM-A9-25 ng/mL CCL11 - Sample 3 (Petri Dish 3)	94,997
SIM-A9-25 ng/mL CCL11 - Sample 4 (Petri Dish 3)	116,138
SIM-A9- 50 ng/mL CCL11 - Sample 3 (Petri Dish 4)	90,008
SIM-A9- 50 ng/mL CCL11 - Sample 4 (Petri Dish 4)	99,587
SIM-A9-100 ng/mL CCL11- Sample 3 (Petri Dish 5)	103,100
SIM-A9 - 100 ng/mL CCL11- Sample 4 (Petri Dish 5)	114,697
SIM-A9 - 200 ng/mL CCL11 - Sample 3 (Petri Dish 6)	113,570
SIM-A9- 200 ng/mL CCL11 - Sample 4 (Petri Dish 6)	132,139

Figure 2B: *Maximum RLU Values from SIM-A9 Microglial Cells Stimulated by CCL11 during 2nd day of testing. Cell Supernatants are correlated to the release of H_2O_2 into cell culture media in response to stimulation.*

RLU values from trial 3 of only Supernatant:

Name of Sample	Cell Supernatant (RLU/s)
SIM-A9- 0 ng/mL CCL11 - Sample 5 (Petri dish 1)	76,331
SIM-A9- 0 ng/mL CCL11- Sample 6 (Petri dish 1)	79,863
SIM-A9-12.5 ng/mL CCL11 - Sample 5 (Petri Dish 2)	87,440
SIM-A9-12.5 ng/mL CCL11- Sample 6 (Petri Dish 2)	78,872
SIM-A9-25 ng/mL CCL11 - Sample 5 (Petri Dish 3)	91,217
SIM-A9-25 ng/mL CCL11 - Sample 6 (Petri Dish 3)	118,688
SIM-A9- 50 ng/mL CCL11 - Sample 5 (Petri Dish 4)	114,113
SIM-A9- 50 ng/mL CCL11 - Sample 6 (Petri Dish 4)	100,883
SIM-A9-100 ng/mL CCL11- Sample 5 (Petri Dish 5)	119,872
SIM-A9 - 100 ng/mL CCL11- Sample 6 (Petri Dish 5)	106,731
SIM-A9 - 200 ng/mL CCL11 - Sample 5 (Petri Dish 6)	124,99
SIM-A9- 200 ng/mL CCL11 - Sample 6 (Petri Dish 6)	135,670

Figure 2C: *Maximum RLU Values from SIM-A9 Microglial Cells Stimulated by CCL11 during 3rd day of testing. Cell Supernatants are correlated to the release of H_2O_2 into cell culture media in response to stimulation.*

As seen in the data tables 2A-2C, the production of H_2O_2 in cell supernatant vs. cell lysate greatly differs. Detected luminescence is directly proportional to the quantity of H_2O_2 in

solution with the negative control of 0 ng/mL of CCL11 demonstrating the natural occurrence of H_2O_2 produced by these cells in vitro. It is seen in all data tables that with the increased treatment of CCL11 there is also a corresponding increase in H_2O_2 production. The ROS-GLO™ H_2O_2 Assay can screen for the presence of H_2O_2, not any of the other superoxide species: hydroxyl radical, hydroxide ion, triplet oxygen, superoxide anion, peroxide ion, and nitric oxide. Superoxide species are converted by superoxide dismutase to H_2O_2, and then converted by catalase or glutathione peroxidase to H_2O in normal cellular responses. It should also be noted that there was visible cell death in the samples treated with 200ng/mL of CCL11 (Petri Dish 6) after the 8-hour treatment period in comparison to the others.

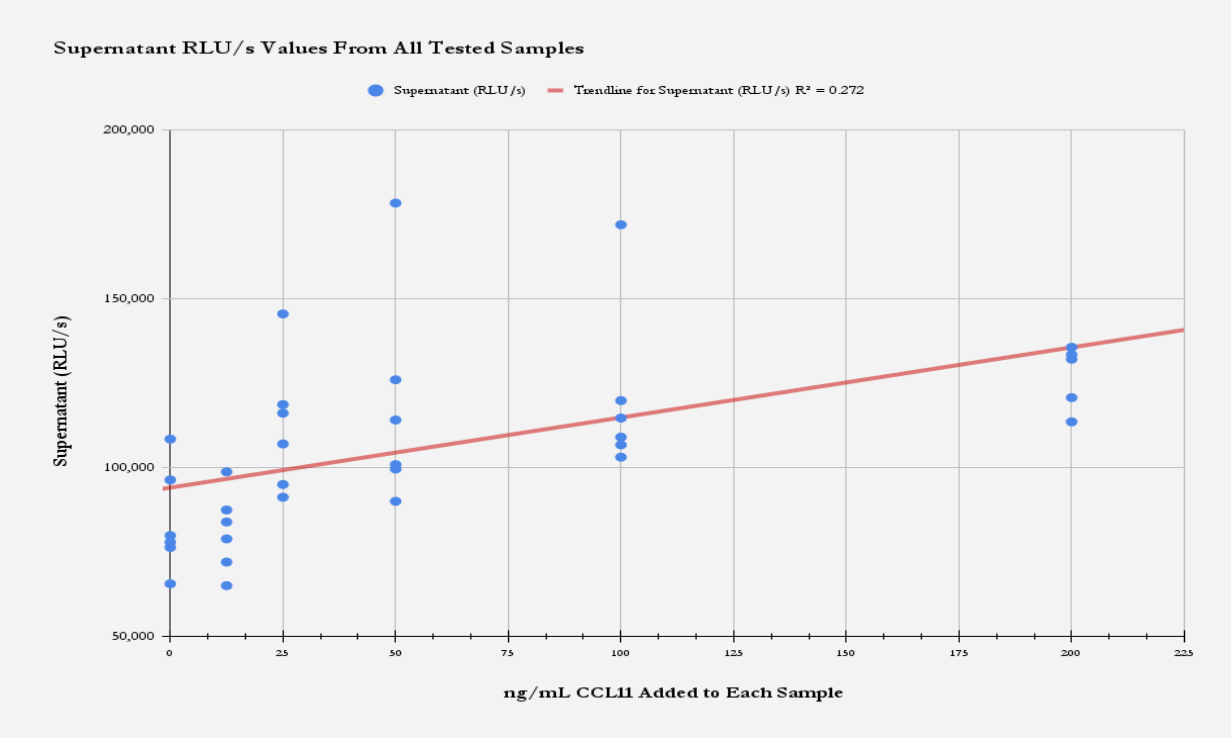

Figure 3A: *All collected Supernatant Data in RLU/s Vs. the amount of CCL11 stimulation in ng/mL. The line of best fit shows an increase in RLU/s values in conjunction with the increase in CCL11 treatment. This directly corresponds to the quantity of H_2O_2 released into the cell culturing medium by SIM-A9 microglial cells in response to CCL11 treatment. R^2 Values = 0.272*

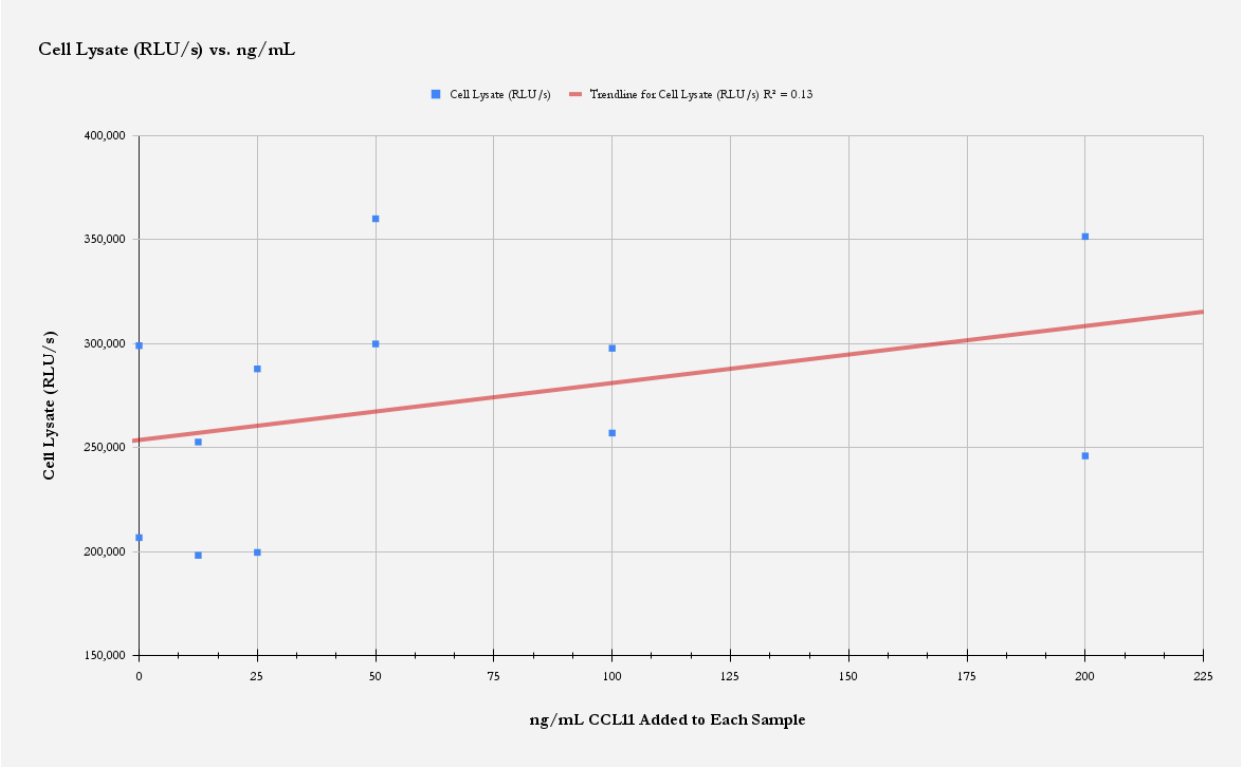

Figure 3B: *All collected Cell Lysate Data in RLU/s Vs. the amount of CCL11 stimulation in ng/mL. The line of best fit shows an increase in RLU/s values in conjunction with the increase in CCL11 treatment. This directly corresponds to the quantity of* H_2O_2 *in each sample produced within SIM-A9 microglial cells in response to CCL11 treatment.* R^2 *Values = 0.13*

From the data plotted in Figures 3A and 3B, the increase in luminescence recorded (RLU/s) is plotted in correspondence to the treatment (0 ng/mL (Control), 12.5 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL). The trendline plotted in red shows the general increase of luminescence that is seen with increasing CCL11 treatment values. Based on the trendline in Figure 3A there was a ROS increase of approximately 40% between the supernatant samples treated with CCL11 at 0ng/mL and those treated with 200ng/mL. These samples represent the quantity of H_2O_2 released by microglial cells in response to the CCL11 treatment. There was also the calculated R^2 value of 0.272 for all the data points in the figure. The R^2 value shows a weak but positive correlation between the supernatant samples, treatment with CCL11,

and the software-generated trendline. Figure 3B represents the increase of intracellular H_2O_2 quantities following CCL11 treatment, and has a corresponding approximate change of 20% from CCL11 treatments of 0ng/mL and 200ng/mL. The graph also displays an R^2 value of 0.13, representing the correlation of data on the graph and their fit to the trendline.

Discussion

Role of ROS in Neurodegenerative Disease

We anticipated that CCL11 would induce the production of ROS in brain macrophages/microglia. CCL11 is an aging-related serum protein, with the levels of CCL11 increasing as we get older. Therefore, we suspect that CCL11-induced ROS would be linked to an increased risk of neurodegenerative diseases in seniors. With the outcome of our data, there was a notable increase in the production of H_2O_2 in both the supernatant and cell lysate samples. Supernatant samples produced the greatest percentage of change from the control sample to that treated with 200 ng/mL of CCL11, with a calculated 40% difference. These results show a positive correlation between the treatment of CCL11 and the quantity of ROS released into the extracellular environment of the in-vitro SIM-A9 cells.

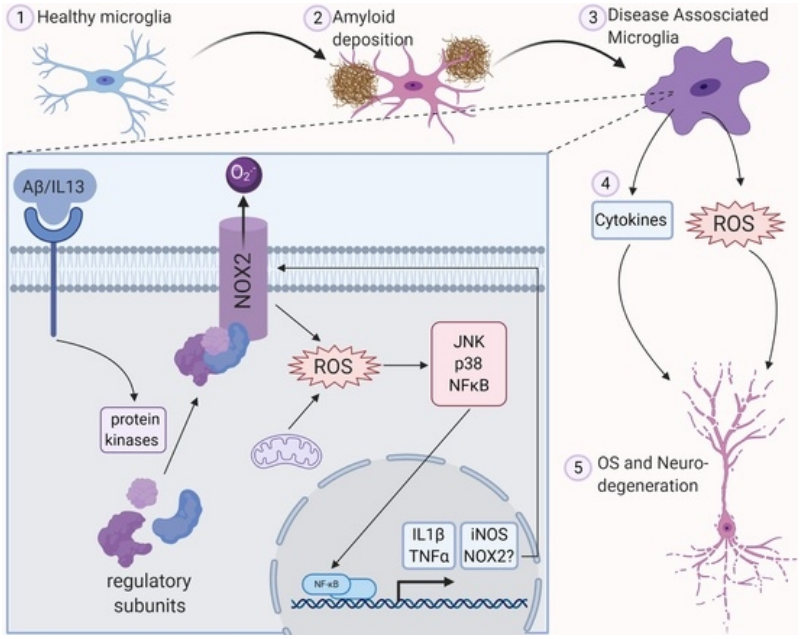

Figure 4A: *Intracellular mechanism by which microglial cells produce ROS via the NOX signaling pathway (Simpson et al., 2020).*

In in-vivo models, it can be predicted that this elevated extracellular ROS would be a factor leading to decreased neurogenesis and neurodegenerative processes within the brain. According to Antunes et al. (2000), due to the high permeability of H_2O_2 across the cell membrane and that most cells within the brain have lower intracellular concentrations of H_2O_2, the gradient of this compound across the cellular membrane is easily established. This means that extracellular ROS has the potential to affect neighboring tissues around where it is produced. Although cells have protective mechanisms against the damaging effects of intracellular H_2O_2, when these levels exceed that of homeostatic conditions, this compound causes oxidative stress to the cell and all of its intracellular components. Figure 4A is a visual reference of the intracellular mechanism by which ROS are produced by microglial cells via the NOX signaling pathway (Simpson et al., 2020). In the research conducted for this project, it was seen that the treatment of 200 ng/mL caused visible cytotoxicity of the SIM-A9 cell culture; Demonstrating,

the effects of this potent compound on the cells that produce it, and the possible consequences of this chemokine in the death of neuronal tissues. With the significant loss of the cells stimulated by 200 ng/mL of CCL11, there is the possibility that greater H_2O_2 levels could have been obtained with an increased survival rate of these stimulated cells. In vivo, microglial cells are responsible for neuronal development and maintenance, upon stimulation with CCL11 it draws to question the ability of these cells to perform this vital function.

RLU/s and ROS

Through the use of the ROS-GLO™ H_2O_2 Assay, proportional H_2O_2 quantification from each sample was able to be recorded in Relative Light Units. Following the calculations and protocol set forth by Promega, the distributing company, the H_2O_2 substrate was added to each sample. Upon reaction of this substrate with H_2O_2, a luciferin precursor was formed. From there the addition of ROS-GLO™ Detection Solution was added to each sample promptly to produce luciferin from the precursor present in the sample.

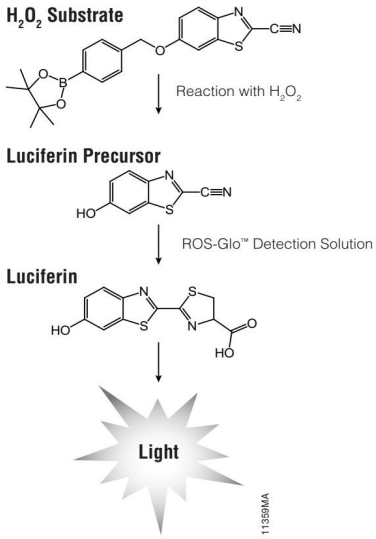

Figure 4B: *Mechanism for the detection of H_2O_2 by the ROS-GLO^{TM} H_2O_2 Assay (Duellman, 2013).*

With the luciferase also present in the sample the corresponding quantity of luciferin was able to be determined through the use of a luminator. Due to the greater presence of luciferase in the sample, the total quantity of luciferin in each sample was recorded in RLU/s by the luminator. The RLU/s produced by each sample is directly proportional to the quantities of H_2O_2 in each sample, allowing for the quantitative analysis of the amount of ROS produced by microglial cells in response to CCL11 stimulation. Higher RLU/s values correspond to more H_2O_2 present in the tested sample. Revealing that greater stimulation of microglial cells by CCL11 corresponds to greater production and release of H_2O_2 into the extracellular environment. With the high permeability of H_2O_2 and other superoxide species through biological membranes, the effect of ROS produced by CCL11-stimulated microglial cells likely has a great influence on surrounding tissues.

Conclusions

Overall, the results of this study can conclude that stimulation of SIM-A9 cell cultures with CCL11 does increase the levels of ROS production by microglial cells in vitro. It also validates the ability of the ROS-GLO™ H_2O_2 Assay to quantify the amount of H_2O_2 in each sample and prove the positive correlation between microglial cells, CCL11 stimulation, and ROS production. The samples stimulated with 200 ng/mL of CCL11 also demonstrated cytotoxic effects of CCL11 on microglial cells, posing potential implications of neurodegenerative disease, as CCL11 levels naturally increase as we age.

Limitations

With the use of the ROS-GLO™ H_2O_2 Assay, only the production of H_2O_2 could be quantified. No other reactive oxygen species were tested for in the sample set used for this research. Also, due to the size and sampling restrictions of the ROS-GLO™ H_2O_2 Assay, the testing of each sample was only performed six times, enabling a general trend to be formed off of the quantitative data.

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