The effect of impaired Cyclooxygenase 2 activity on gene regulation in the developing mouse brain and the role of PGE_2 in oxidative stress production in differentiated neuronal cells

Sasha Udhesister

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN KINESIOLOGY AND HEALTH SCIENCES

YORK UNIVERSITY

TORONTO, ONTARIO

January 2021

Abstract

Prostaglandin E2 (PGE_2) is a signaling molecule derived from the lipid membrane through the enzyme cyclooxygenase–2 (COX-2). These studies aim to investigate how changes in COX-PGE_2 signalling can influence mouse gene expression and alter oxidative stress in neuronal cells. In study 1 we use microarray analysis to identify differentially expressed genes among males and females at embryonic day 16 (E16) and 19 (E19). Bioinformatics software outlined genes involved in mitochondrial function, inflammatory responses and synaptic plasticity. In study 2, differentiated Neuroectodermal (NE-4C) stem cells were treated with two concentrations of PGE_2. Fluorescence microscopy with MitoSox Red was used to measure superoxide production. Both concentrations of PGE_2 significantly increased superoxide production in a dose-dependent manner. In summary, these results indicated that altered levels of PGE_2 can result in abnormal expression of important developmental genes involved in the mitochondrial function, as well as production of reactive oxygen species (ROS).

Acknowledgements

First, I would like to extend my sincere thanks to my research supervisor, Dr. Dorota A. Crawford. Over the last two years she has always supported and guided me. I am truly grateful for all of her patience and encouraging words along the way. She has helped me become a better scientist. I would also like to thank my committee members, Dr. Christopher G. R. Perry and Dr. Terrance J. Kubiseski. I would especially like to thank Dr. Perry for taking the time to meet with me and discuss my analysis as well as provide me with feedback along the way.

Additionally, I would like to thank my lab members, who have supported me since day one. Thank you Ashby for training me and always answering any questions I had. You were always there to lend a helping hand whenever I needed you. I would also like to extend my appreciation to Keenan for always being there for me and for continuously providing me with advice. Sarah and Isabel, thank you both for welcoming me into the lab and for always supporting me. It means a lot to me that both of you are still looking out for me all the way in B.C. Shalini and Kelly, I am really glad you both joined the lab and thank you for helping me in any way possible. I would also like to thank Shalini for her hard work on the Nitric oxide detection, it was great working with you. I would also like to thank Denis for training me on the cell culture and for always answering all of my questions. It was a wonderful experience working alongside each of you, and you have each made my experience in the lab amazing.

Last be certainly not least, I would like to thank all of my cheerleaders behind the scene. I would especially like to thank my Mom, Dad and Brother, who have always supported me through everything. Mom and Dad, I appreciate all of your support and love, I hope you know that I always strive to be my best because of the amazing example you have set for me. You both work endlessly to ensure that Dylan and I have everything we need, and your unconditional love and support has always motivated me to make you proud. I appreciate all that you do for me. Dylan, thank you for always being a listening ear and for always saying the right thing. Even though you're my younger brother, I still look up to you and respect you. You are the best brother I could ask for and you really have encouraged me to always be my best. A special thanks to my amazing best friend for always motivating and supporting me since day one. It means so much that you're always cheering me on and looking out for me. Finally, I would like to thank my friends and family who have also encouraged me along the way. I love you all and without each of you, this would not be possible.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

AA – Arachidonic acid

Acvr1- Activin A Receptor Type 1

AMPAR - Alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors

AutDB- Autism database

ASD – Autism spectrum disorder

ATP5e- ATP Synthase Epsilon Chain E

CNS – Central nervous system

COX1 – Cyclooxygenase-1

COX2 – Cyclooxygenase-2

Cyfp1- Cytoplasmic FMR1 Interacting Protein 1

DAF2-DA - 4,5 Diaminofluroescein Diacetate

E16/19- Embryonic day 16/19

ETC- Electron transport chain

eNOS – Epithelial Nitric Oxide Synthase

EP - E-Prostanoid Receptors

EiF3C- Eukaryotic Translation Initiation Factor 3 Subunit C

EiF4A2- Eukaryotic Translation Initiation Factor 4 Subunit A2

EiF4e- Eukaryotic Translation Initiation Factor 4E

FBS – Fetal Bovine Serum

FMRP1- FMRP Translational Regulator 1

GRiA2- Glutamate Receptor Ionotropic AMPA Type 2

Grinb2- Glutamate Receptor Ionotropic NMDA Type 2B

iNOS – Inducible Nitric Oxide Synthase

O_2^{-} Superoxide anion

KEGG-Kyto Encyclopedia of genes and genomes

KI – Knock-In

LPS – Lipopolysaccharide

LTP – Long-term potentiation

MEFC2- Myocyte Enhancer Factor 2C

MEM – Minimum essential media

NdufA3-NADH: ubiquinone oxidoreductase subunit A3

NdufC2- NADH: ubiquinone oxidoreductase subunit C2

NdufB10- NADH: ubiquinone oxidoreductase subunit B10

NE-4C – Neuroectodermal stem cells

nNOS – Neuronal nitric oxide synthase

NO – Nitric oxide

NOS – Nitric oxide synthase

Nfib- Nuclear Factor IB- Type

Nfix- Nuclear Factor IX- Type

PBS – Phosphate-buffered saline

PCR – Polymerase chain reaction

PGE_2 – Prostaglandin E2

PKA – Protein kinase A

Pkcb- Protein Kinase C Beta

PLA2 – Phospholipase A2

RNS – Reactive nitrogen species

ROS – Reactive Oxygen Species

Sdhc- Succinate Dehydrogenase Complex Subunit C

Tgfbr1- Transforming Growth Factor B Receptor 1

Tgfbr2- Transforming Growth Factor B Receptor 2

TRP53- Tumor protein 53

TSC 1- Tuberous Sclerosis 1 Protein

TSC2- Tuberous Sclerosis 2 Protein SFM – Serum free media

Wnt - Wingless-related integration site

Chapter 1- Introduction and Background

1.1 PGE_2 role in Autism

1.1.1 PGE_2 and healthy brain development:

Omega-6 linoleic acid is a precursor molecule for Arachidonic acid (AA), which can be cleaved from the phospholipid membrane via phospholipase A2 (PLA2), due to increased neurotransmitter binding (Farooqui et al., 2007). This can also occur due to environmental factors, which can lead to increased cytokine expression (Farooqui et al., 2007). AA is an important precursor molecule because it can be further metabolized into bioactive molecules known as eicosanoids. Some examples of eicosanoids include prostaglandins, thromboxanes, leukotrienes, lipoxins, and cannabinoids (Bell et al., 2004).

In order for AA to be converted into eicosanoids, it must first be converted into PGH_2 by Cyclooxygenase 1 (COX-1) and Cyclooxygenase 2 (COX-2), as a result of several oxygen-driven reactions (Lee et al., 2017). Following this, several prostaglandins can be generated, including PGE_2, PGF_2, PGD_2, and thromboxane A2 (Lee et al., 2017). The major lipid molecule in the nervous system is PGE_2. The COX- PGE_2 pathway regulates processes such as gene expression through the activation of the G-protein-coupled EP receptors (Furnuyashiki and Narumiya, 2011). Once PGE_2 is synthesized, it binds and activates, one of several E-prostanoid receptors, known as EP1-4 (Lawrence, 2010). Each receptor has a specific function. For example, EP1, is known to play a role in Ca^{2+} concentrations, while EP2, EP3 and EP4, play roles in cAMP and PKA signalling (Lawrence, 2010). A brief overview of the metabolism of PGE_2 can be found in Figure 1.

1.1.2 Abnormal PGE_2 signaling and ASD

Abnormalities in fatty acid metabolism have been seen to have characteristic effects in individuals with autism spectrum disorders (ASD) (Yehuda et al., 1999). These imbalances can have effects due to the fact that these fatty acids can play crucial roles in the development, maintenance and function of the nervous system (Lawrence, 2010). Abnormal levels of fatty acids can be a result of many different factors, such as environmental factors (Yehuda et al., 1999). A very common type of abnormality which is found in individuals with ASD, are imbalances to omega-6 (Yehuda et al., 1999). Omega-6 imbalances have been noted to be found alongside several ASD phenotypes (Lawrence, 2010).

Abnormalities in the COX2-PGE_2 signaling pathway due to genetic and environmental factors can also lead to ASD. There has also been substantial research investigating the link between abnormal lipid signalling and ASD (Sang and Chen, 2006). For instance, there has been evidence linking the COX2- PGE_2 pathway and many functions involved in healthy brain development. Some functions which have been noted to be affected by disruptions in the COX2-PGE_2 pathway include, dendritic spine formation, learning and memory, and synaptic plasticity, all of which have actually been associated with ASD as well (Chen et al., 02, Chen and Brazan, 2005, Burks et al., 2007). One factor which can lead to ASD is an abnormal ratio of AA to Omega 3 and 6 fatty acids. This can result in increased PLA2 activity and a decrease in AA, as well as an overall increase of PGE_2 in the blood of human patients with ASD (Bell et al., 2010, Jory, 2016, Tostes et al., 2013). Also, a genetic factor which can lead to increased risk of ASD is involving the *Ptgs2* gene which encodes for COX-2. A polymorphism in this gene has been seen to connect to ASD behaviours (Yoo et al., 2008). Additionally, evidence indicates that the misuse of misoprostol is linked to ASD. Misoprostol is a PGE_2 analogue that can bind to the EP

receptors. When misoprostol is ineffectively used in an attempt to terminate a pregnancy, it has been associated with an increased risk of ASD in the offspring (Bandimal, 2003, Schuler et al., 1999, Pastusrak et al., 1998). Our review article describes the link between various abnormalities in the COX2-PGE_2 pathway and ASD. For instance, we noted that environmental factors such as, inflammatory responses, oxidative stress, pesticides, over-the-counter drugs, are all known to influence the level of PGE_2, increase the vulnerability of the brain to the effects of PGE_2 and have been associated to ASD (Tamiji and Crawford, 2010, Wong et al., 2014).

1.1.3 Role of COX-1 and Cox-2 in the brain

There is also evidence connecting the COX2-PGE_2 pathway with crucial aspects of brain development. As mentioned previously, there are two forms of COX which play a role in PGE_2 synthesis, COX-1 and COX-2. COX-1 is constitutively expressed in the brain and is primarily found in microglia and is seen to be upregulated in many biological processes (Amateau and McCarthy, 2004, Kirkby, 2012). On the other hand, COX-2 is found in an inducible form as an inflammatory response in many tissues, such as kidney and intestinal tissues (Yamagata et al., 1993). However, in the brain COX-2 is constitutively expressed primarily in glutamatergic neurons, the cerebral cortex, hippocampus, cerebellum, and spinal cord (Hewett et al., 2016, Kaufmannet al., 1996). There have been many research articles noting that COX2-PGE_2 signalling plays a role in healthy brain development. For instance, it was observed that COX-1 and COX-2 expression was seen to be increased in the fetal brain, thus indicating that these enzymes play a role in brain development (Gersting et al., 2009). Additionally, the COX2-PGE_2 pathway has been connected to embryonic development and neurogenesis. For instance, it was observed that there is a spike in PGE_2 levels and EP receptor expression during embryonic

development, coinciding with the onset of neurogenesis (Hatae et al., 2002, Tamiji and Crawford, 2010).

1.1.4 COX-PGE_2 connection to ASD

Research from our lab has indicated molecular evidence to link disruptions in the COX2-PGE_2 pathway with brain development and ASD. For example, *in vitro* studies in our lab have demonstrated that treating cells with increased concentrations of PGE_2, have altered many cellular functions. There has been evidence that treating neuronal cells with PGE_2 has shown altered intracellular calcium levels in the growth cones and cytosol of cells, hindrance of neurite outgrowth, an increase in migration and proliferation of neural stem cells, as well as accelerated neuronal differentiation (Tamiji and Crawford, 2010, Davidson et al., 2016, Wong et al., 2014). Furthermore, a study in our lab showed that treating NE-4C cells with PGE_2 altered Wnt-dependent migration and proliferation of the cells (Wong et al., 2014). This is an important finding since Wnt has been shown to play a key role in prenatal brain development, indicating that alterations in Wnt due to PGE_2 treatment could have lasting effects on neurodevelopment (Wong et al., 2014).

In vivo studies were also conducted in our lab to highlight the effects of disrupting the COX2-PGE_2 pathway in an animal model and how this can relate back to ASD. Studies were done using a COX-2 deficient mouse model and a PGE_2 injected mouse model to investigate how altered levels of PGE_2 can affect the developing mouse brain. One study in our lab was conducted using a COX-$2^{-/-}$ knockout (KO) mouse model to conduct a whole genome microarray analysis on mice at embryonic day 16 and embryonic day 19 (E16 and E19). This analysis indicated that the COX-2 deficiency in males and females led to the dysregulation of many genes, including those associated with ASD and many other neurological processes such as

synaptic plasticity, long-term potentiation, and axonal guidance (Rai-Bhogal et al,. 2017; Rai-Bhogal et al, unpublished).

Two other studies in our lab focused on how altered levels of PGE_2 led to behavioural abnormalities in mouse offspring, which are characteristic of ASD. The studies used a COX-2^- knockin (KI) model and a PGE_2 injected model. The study using the COX-2 KI model concluded that ASD behaviours were more expressed in males rather than females (Wong et al., 2017). The behaviours demonstrated by the mice were associated with increased anxiety, hyperactivity, motor defects, and decreased sociability, all of which are associated with ASD behaviours (Wong et al., 2017). Furthermore, another study in our lab showed that if pregnant mice are injected with PGE_2 at embryonic day 11 (E11), similar behaviour defects are noted in offspring (Wong et al., unpublished). These studies are similar to another study where the COX-2 deficiency also led to ASD-like behaviours primarily in rat offspring (Dean et al., 2012). This study also showed that male rats with the COX-2 deficiency also exhibited changes in sensory integrations and social interactions, both of which are phenotypic characteristics of ASD (Dean et al., 2012). Therefore, both of these studies indicate that prenatal irregularities in the COX2-PGE_2 pathway can have lasting effects postnatally, which may contribute to ASD.

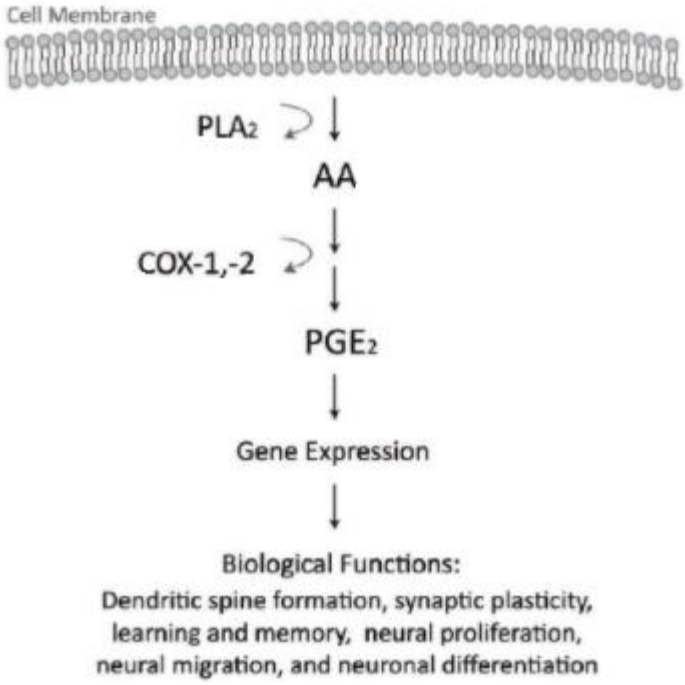


Figure 1. Synthesis of PGE_2. Arachidonic acid can be converted into various prostaglandins. One of the most active prostaglandins is PGE_2, which can activate four types of EP receptors, known as EP1, EP2, EP3, and EP4. The activation of these receptors can lead to many physiological and pathological processes by altering gene expression (Wong et al., 2017).

1.2 Oxidative Stress

1.2.1 What is Oxidative stress?

Over the years, there has been research suggesting that oxidative stress and PGE_2 are connected. Much of this research has been done to show that oxidative stress can influence the production of PGE_2, and recently there has been research indicating a possible feedback loop, where changes in PGE_2 can also lead to alterations in the amount of oxidative stress within cells (Akyol, 2004, Black et al., 2011. Kosuge et al., 2020). Within cells there is an equilibrium present in which reactive oxygen and nitrogen species are regulated by antioxidants (Chahum, 2016). However, there may be times when the amount of reactive species is greater than the antioxidant molecules are capable of lowering, leading to redox stress (Lushchak, 2014). One very common type of this stress is known as oxidative stress. This is usually caused by oxygen molecules being converted into free radicals or non-radical oxidants, which can have detrimental effects on the cells. These oxidants are usually generated from the mitochondria, through leaks in the electron transport chain, or through oxygen conversion elsewhere in cells, such as via enzymes (Seis et al., 2017). In fact, about 90% of free radicals generated, are derived from the mitochondria (Lushchak, 2014). The first conversion which happens to oxygen is the production of superoxide anion (O_2^-) (Islam, 2016). From there many other oxidants can be produced, such as hydroxyl radical and hydroxyl anion (Islam, 2016)). Superoxide is one of the most common types of reactive oxygen species (ROS). Also, the enzyme nitric oxide synthase (NOS), can convert nitrogen into nitric oxide (NO), a very common reactive nitrogen species (RNS) (Costa et al., 2016).

Usually, the concentration of these redox by-products fluctuates within a normal range, which is regulated by antioxidant activity. However, for oxidative stress to occur there is an

imbalance between the generation and elimination of the redox oxidants (Drose and Brandt, 2012). Therefore, one of the better definitions for oxidative stress is, when the concentration of the normal amount of ROS is chronically enhanced, this can lead to detrimental effects on the cell (Figures 2 and 3) (Bigarella et al., 2014). Some of these effects include, disturbing cell respiration, metabolism, gene expression and eventually cell death (Bigarella et al., 2014).

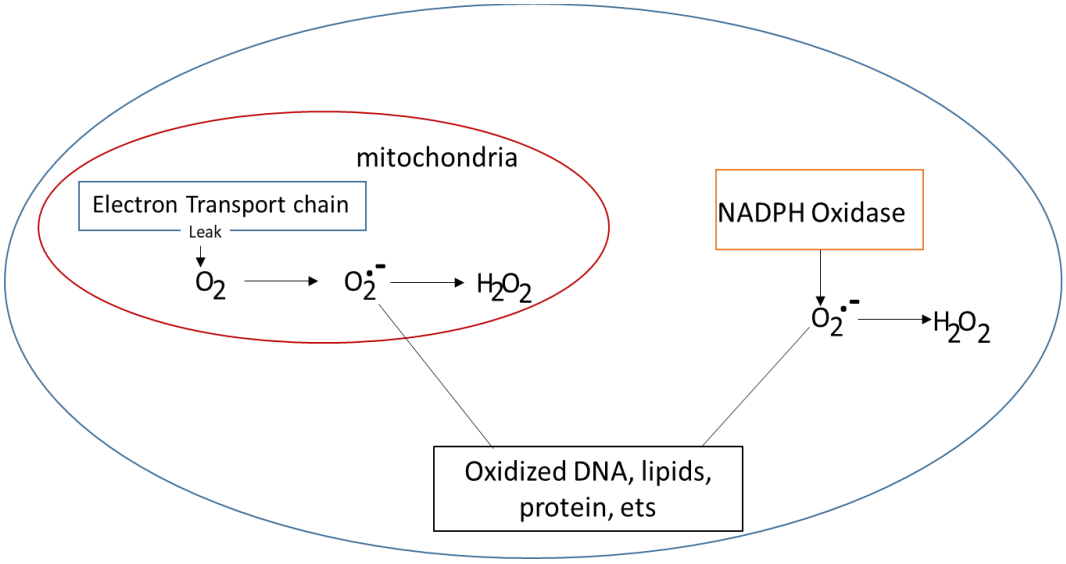


Figure 2. ROS generation and effects on the cell. ROS generation can start via leaks from mitochondria or enzymes such as NADPH oxidase. From here superoxide (O_2^-) can be produced, which can be converted into other radicals and non-radical oxidants, such as hydrogen peroxide, and then hydroxyl radical. The production of such oxidants can have many effects within the cell. For example, proteins, lipids and DNA can be oxidized, leading to many changes in the cell.

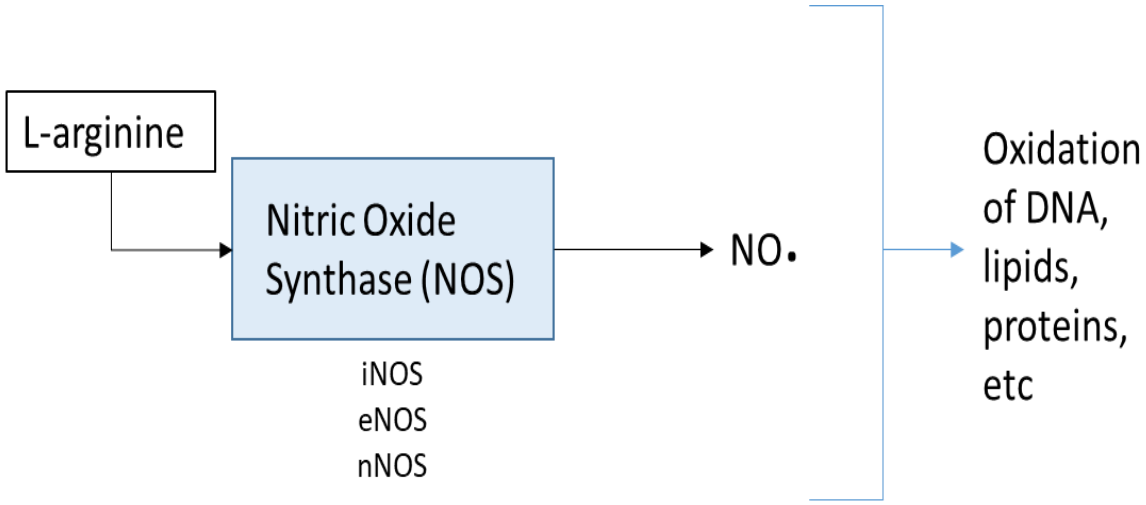


Figure 3. RNS production and effects on oxidation. Nitric oxide can be generated from the enzyme nitric oxide synthase, which can be activated by O_2^-. Increased amounts of nitric oxide can lead to various affects in the cell such as, protein, lipids and nucleic acid oxidation.

1.2.2 Oxidative stress in the Brain

Interestingly, one of the most vulnerable areas to redox is the brain (Chahum, 2006). The main reason for this is the lack of antioxidants in the brain (Cobley et al., 2018). Other reasons include the fact that the brain requires much more energy than other parts of the body, and the brain has higher lipid and iron concentrations (Cobley et al., 2018). Most of the energy used in the brain is burned by the neurons. Neurons in particular lack a very important antioxidant known as glutathione (GSH), therefore leaving neurons and the brain vulnerable to oxidative stress because they lack the essential ability to detoxify ROS (Cole et al, 2016). Another interesting fact is that the brain is more susceptible to ROS during early development and this phenomena has been linked to many neurological disorders (Cole et al, 2016).

1.2.4 Oxidative stress in ASD

As mentioned previously, glutathione (GSH) is an antioxidant that works as a major redox buffer (Robb et al, 2018). There is a specific ratio within an organism that must be maintained between GSH and its oxidized form, GSSG (Dominko and Dikic, 2018). At basal concentrations, GSH/GSSG work as a redox scavenger and helps maintain basal levels of radicals. However, at times of chronic ROS exposure, there is less GSH, so the redox homeostasis is altered (Dominko and Dikic, 2018)). In autism spectrum disorders, it has been shown that there are reduced amounts of GSH, but increases in the GSSG concentrations (Robb et al., 2018). This is similar to what we expect to see with increased ROS, suggesting that increased ROS is characteristic of ASD.

Also, it has been seen in samples of blood plasma from children with ASD, that there is increased NO production (Yui et al., 2016). Increased oxygen radicals have been shown to

increase inflammation, damage the cell membrane, and lead to cell death (Yui et al., 2016). With that, there is a lot of evidence to suggest that chronic oxidative stress is a characteristic of ASD.

1.2.5 Oxidative Stress and PGE_2

The connection between oxidative stress and PGE_2 is still growing. There is some evidence to suggest that ROS and RNS lead to PGE_2 production, as well as research showing that PGE_2 can also lead to ROS and RNS production, which would suggest a type of positive feedback loop. There are studies which indicate that PGE_2 influences ROS and RNS production, however in many of these studies, non-neuronal cells are investigated. Based on this evidence, I will be investigating the role PGE_2 plays on ROS and RNS production in neuronal cells.

For starters, a study investigated how ROS induced from mechanical injury to cultured human Keratinocytes, affected PGE_2 production. Here, it was seen that over time as more ROS was produced, by increased injury to the keratinocytes, more PGE_2 was produced as well. PGE_2 was produced in an exponential manner until the cells became saturated and a plateau in PGE_2 production was observed (Black et al., 2011). This suggests that ROS played a role in PGE_2 generation in human keratinocytes.

Furthermore, in a paper looking at the production of PGE_2 in LPS treated rat microglia, it was shown that the LPS exposure led to a significant PGE_2 production increase (Wang, et al., 2004). Also, it was seen that by inhibiting NADPH oxidase, there was a significant decrease in PGE_2 production. LPS is also well-known to increase ROS production in many ways, including via the enzyme NADPH oxidase (Akyol, 2004). Therefore, this adds to the idea that LPS induces ROS, through NADPH oxidase activity, which in turn leads to PGE_2 production (Wang, et al., 2004). This indicates that there is a connection between ROS production and PGE_2 production.

Also, there is a recent study which showed that increased levels of PGE_2 also increased the production of ROS, which in turn led to a greater degree of neurotoxicity in motor neurons, similar to the effects seen in ALS (Kosuge et al., 2020). NSC-34 cells were plated and used to conduct ROS detection experiments (Kosuge et al., 2020). In this paper, researchers also investigated how the antioxidant N-acetyl cysteine (NAC), can influence ROS production. It was observed that PGE_2 led to increased ROS production and increased levels of neurotoxicity, but treatment with NAC led to much less neurotoxicity within cells. This exemplifies that PGE_2 does indeed participate in ROS generation, suggesting the existence of a feedback loop between the production of PGE_2 and ROS. This is a concept that will be further investigated in our neuroectodermal stem cells.

Additionally, there are many studies which investigate the connection between NO and PGE_2. For instance, in a study looking at human granulosa cells cultured from women undergoing IVF treatment, it was observed that increased NO production, via the use of the NO donor SNAP, led to a significant increase in PGE_2 production (Fang et al., 2015). This is an example of how treating cells with NO can influence PGE_2 production.

Interestingly, in a study looking at how treatment with norepinephrine alters NOS activity and PGE_2 production in the hypothalamus of mice, it was observed that treatment with norepinephrine led to increased levels of NO production and increased levels of PGE_2 production in medial hypothalamic slices (Retton et al., 1992). Furthermore, inhibiting NOS activity with NMMA treatment, led to significantly less PGE_2 production, compared to basal levels of PGE_2 production (Retton et al., 1992). These results indicate that in the medial hypothalamic fragments, NO is one factor responsible for abnormally increased PGE_2 production via NO activity (Retton et al., 1992).

Furthermore, there has also been evidence that PGE_2 can influence the production of NO. A recent study indicated that PGE_2 influences the function of iNOS, thus altering the production of NO in murine cells (Cheng et al., 2011). There were also results indicating that nNOS can also be altered by PGE_2, which also altered NO production (Cheng et al., 2011). Based on these findings, there is evidence that there is indeed a feedback loop between nitric oxide and PGE_2.

Based on the available evidence from non-neuronal cells described above, it appears that various forms of ROS and RNS can influence the production of PGE_2. Two types of oxidants which are involved in the production of PGE_2 are superoxide and nitric oxide.

The free radical, superoxide, also plays a role in PGE_2 production. Superoxide leaked from the mitochondria has been seen to induce COX-2 activity and PGE_2 production (Blanco et al. 2008). Mitochondrial dysfunction has many implications on cell function, including PGE_2 production. It has been seen that as the mitochondria continues to leak superoxide, it is also seen to increase the level of mitochondrial dysfunction within cells, as well as alter PGE_2 production (Blanco et al., 2008). This combination of increased ROS, mitochondrial dysfunctional, and abnormal levels of PGE_2, can have detrimental effects on cells (Blanco et al., 2008). In a recent study it was seen that PGE_2 can also play a role in mitochondrial dysfunction by altering the membrane potential of the mitochondrial membrane, leading to increased production of ROS, including superoxide (Corrado et al., 2018).

When it comes to the PGE_2 pathway, nitric oxide has been seen to play a critical role in its signaling. It has been observed that nitric oxide, derived from NOS, can bind to specific receptors on the COX-2 enzyme, which can influence PGE_2 production (Sangwon, 2014). It has also been observed that NO synthesized by two isforms of the NOS enzyme, nNOS and iNOS, can bind to the COX-2 enzyme, which in turn influences the production of many prostaglandins,

including PGE_2 (Sangwon, 2014). In fact, a recent study has indicated that the opposite pathway is true too, where PGE_2 influences the function of iNOS, thus altering the production of NO in murine cells (Arcoleo et al., 1995).

As seen from the examples above, superoxide and NO can alter PGE_2 production by influencing COX-2 activity and the pathway by which PGE_2 is produced. However, there have been very recent studies that indicate that a feedback loop may be present, where PGE_2 levels can alter the production of superoxide and NO. Therefore, in this study I will investigate if this feedback loop is present in differentiated neuronal cells as well.

1.2.6 Other Roles of Nitric Oxide

As mentioned previously, nitric oxide contributes to oxidative stress conditions in cells, but there are also positive effects associated with NO. NO plays a role in learning and memory and contributes to neurotransmission (Akyol, 2004). NO diffuses from the post synaptic membrane to the presynaptic neuron's membrane, leading to neurotransmission (Akyol, 2004). Due to nNOS producing NO which crosses into the presynaptic neuron, guanylyl cyclase becomes activated, leading to cyclic guanosine-monophosphate (cGMP) binding to downstream receptors, such as cGKI, which promotes presynaptic neurotransmitter release (Vincent, 2010). Therefore, NO plays a crucial role in proper neuron function and influences many downstream pathways (Vincent, 2010).

Additionally, NO plays a role in long term potentiation (LTP), resulting in synaptic plasticity and learning and memory (Esplugues, 2002). In order for LTP to occur, glutamate must bind to NMDA receptors on the post synaptic neuron, which sparks many downstream events, including nNOS producing NO (Jung et al., 2013). Following this, increased levels of NO also

lead to increased glutamate production in the presynapse, resulting in a sort of positive feedback loop, also strengthening LTP (Jung et al., 2013)

Therefore, due to the many roles NO plays in the CNS, there must be a balance which is maintained within cells. If this balance becomes disturbed, there may be many detrimental effects to cells and the brain, such as discrepancies in LTP, learning and memory, and even oxidative state (Esplugues, 2002).

Chapter 2- Objectives and Hypothesis

2.1 General Objective

The overall objective of this research is to examine the effect of altered COX2-PGE_2 signalling on the development and function of the nervous system. My aim is (1) to evaluate if reduction in PGE_2 levels affect prenatal brain development by investigating key biological processes, such as mitochondrial function, inflammatory response and synaptic plasticity, using whole genome microarray analysis data generated in our lab from male and female mice lacking cyclooxygenase-2 (COX-$2^{-/-}$) as a new model systems of ASD (Study 1) and (2) whether exposure to increased PGE_2 levels leads to oxidative stress using our *in vitro* neuronal cell model system (Study 2).

2.2 Study 1: Identification of genes associated with mitochondrial function, inflammatory response, and synaptic plasticity using the whole genome microarray data from male and female COX2-/- offspring

2.2.1 Objective

The objective of this study is to examine differentially expressed genes of COX-2 knockout (COX-$2^{-/-}$) offspring which are associated with mitochondrial function, inflammatory responses, and synaptic plasticity, and determine their association to ASD. My goal is to identify which ASD-risk genes belong to important developmental gene networks and identify if there are any stage and sex-dependent differences.

2.2.2 Rationale

Previous research in our lab has indicated several trends from previous microarray studies. For example, it was observed that males had more differentially expressed genes at E16 than E19, but females had similar amounts of differentially expressed genes at both stages. (Rai-Bhogal et al,. 2017; Rai-Bhogal et al, unpublished). Also, females were observed to have more genes which were differentially expressed at both stages. In fact it was observed that a majority

of these genes which were differentially expressed at both stages in females, shifted from a downregulation at E16 to an upregulation at E19 (Rai-Bhogal et al,. 2017). This indicates that in females there is an increased risk of a differentially expressed genes retaining abnormal expression throughout neurogenesis until birth (Rai-Bhogal et al, unpublished). Additionally, it was observed that more genes which were differentially expressed in males were connected to neurodevelopmental disorders compared to those found in females (Rai-Bhogal et al,. 2017; Rai-Bhogal et al, unpublished). Lastly, there were 44 ASD-risk genes identified in males compared to only 6 ASD-risk genes differentially expressed in the female data (Rai-Bhogal et al,. 2017; Rai-Bhogal et al, unpublished). There were also behavioural abnormalities found to be associated with a COX-2 deficiency seen to also be associated with ASD. For instance, a study from our lab using a COX-2^- knockin model concluded that ASD behaviours were observed more often in males rather than females (Wong et al., 2017). The behaviours demonstrated by the mice were associated with increased anxiety, hyperactivity, motor defects, and decreased sociability, all of which are associated with ASD behaviours (Wong et al., 2017).

In this study, I used the microarray data to investigate differentially expressed genes associated with mitochondrial function, inflammatory responses, and synaptic plasticity within our *COX-2*$^{-/-}$ mice, and to research if there are stage and sex differences in the expression of these genes. This model was used due to the fact that there has been an abundance of evidence in recent years, connecting abnormalities in PGE_2 and ASD (Tamiji and Crawford, 2010, Dean et al., 2012, Wong et al., 2017, Rai-Bhogal, 2017).

Furthermore, genes associated with these three functions are of interest because many studies have linked abnormalities in mitochondrial function, inflammatory responses and synaptic plasticity with ASD. For instance, abnormal mitochondrial function can lead to

increased levels of oxidative stress and further mitochondrial dysfunctions. Both of these have negative effects on a developing fetus and have been linked to ASD (Blanco et al., 2008). In fact, literature has shown that environmental factors such as oxidative stress can further alter PGE_2 and lead to ASD (Frustaci et al., 2012, Valko et al., 2005).

Additionally, disruptions in the inflammatory response mechanism can lead to detrimental effects on cells and has been linked to ASD. For example, studies have shown that genes involved in neuroinflammatory responses are found to be dysregulated in brain tissue samples of children with ASD (Ralnigles et al., 2011). Another study has indicated that certain inflammatory markers such as microglia and cytokines are seen in much higher volumes in brain samples of children with ASD (Allely et al., 2013, Elias et al., 2015). As mentioned previously, the COX-2 inducible form plays a key role in inflammatory responses, so deficiencies in COX-2 are expected to greatly alter inflammatory responses (Blais et al., 2005).

Furthermore, disruptions in synaptic plasticity are also associated with ASD. For instance, studies have indicated that abnormalities in synaptic plasticity can lead to disruptions in neural circuits which are involved in language and social behaviours, characteristic in ASD (Gilbert et al, 2014, Kelleher et al., 2005). Two studies in our lab, previously using a COX-2 deficient mouse model as an ASD mouse model, have indicated that synaptic plasticity can be altered by this deficiency. For example, previous microarray analysis identified many differentially expressed ASD-risk genes which also had ties to synaptic plasticity (Rai-Bhogal et al., unpublished). Furthermore, in the COX-2 knockin model, genes associated with ASD behaviours were identified as differentially expressed and many of them also played a role in synaptic plasticity.

Also, many genes which are primarily associated with mitochondrial function, inflammatory responses, and synaptic plasticity are also associated with genes involved in other processes as well. The stage at which the genes are differentially expressed is important to evaluate because ASD is a known neurodevelopmental disorder. Therefore, by identifying at what point in development specific genes are dysregulated, we can gather insight into how differential gene expression at different stages can have implications postnatally as well. Additionally, investigating sex differences is valuable as well due to the fact that many neurodevelopmental disorders, such as ASD are characterized by a sex-bias, where males are more likely to have the disorder. Looking at sex differences between gene regulation patterns may provide insight which may be connected to the sex-bias observed in individuals with these neurodevelopmental disorders.

2.2.3 Hypothesis

I hypothesize that there will be genes associated with mitochondrial function, inflammatory responses, and synaptic plasticity, which are differentially expressed in the E16 and E19 *COX-2*$^{-/-}$ offspring. These genes will also be expressed differently based on the sex of the offspring. Finally, genes identified from the microarray analysis to be primarily a part of one functional group, will also influence genes from the other two groups of interest. Also, I hypothesize that the mitochondrial, inflammatory and synaptic plasticity genes identified in this study in *COX-2*$^{-/-}$ offspring using David Bioinformatics and KEGG databases, are causative genes in ASD and also other neurological disorders.

2.2.4 Methodology

Mitochondrial, inflammatory response, and synaptic plasticity genes identified to be differentially expressed in the microarray data were screened through several different types of databases such as DAVID, GeneMania, KEGG, and Cytoscape, in order to identify networks

and biological functions which these genes influence. Due to the interest of these genes and their connection to ASD and other disorders, the stage and sex at which the dysregulations occurred were also be scrutinized.

2.2.5 Experimental Model system

Pregnant females which were heterozygous for the *COX-2* gene were obtained from Taconic Bioscience (Wong et al., 2017, Rai-Bhogal, 2017, Rai-Bhogal, unpublished). These mice were bred with homozygous knockout males to get offspring at the E16 and E19 stages, which are homozygous *COX-2*$^{-/-}$. Wild-type mice are also bred to also provide E16 and E19 samples. In order to determine the embryonic day, a vaginal plug is used to identify Day 1. On day 16 and 19, samples are collected from the *COX-2*$^{-/-}$ and wild-type mice, and they are placed in a TRIzol solution. Three samples from the *COX-2*$^{-/-}$ and wild-type mice are collected. The COX-2$^{-/-}$ mouse model is a suitable model for this experiment because it has been used in many other papers as a means to investigate the molecular mechanisms of the genetic deficit in COX-2, to investigate how this deficit relates to ASD (Liu et al., 2017, Kwan et al., 2016). Breeding of the animals, collection of RNA and microarrays analysis was conducted in our lab by previous students (Wong et al., 2017, Rai-Bhogal, 2017, Rai-Bhogal, unpublished).

2.3 Study 2: *The effects of* PGE_2 *on the production of superoxide and nitric oxide, in differentiated neuronal cells*

2.3.1 Objective

The objective of this study is to determine if PGE_2 alters the concentration and rate of superoxide and nitric oxide production, within differentiated neuronal cells. Furthermore, we aim to investigate whether these changes induced by PGE_2 are dose-dependent.

2.3.2 Rationale

A mentioned above, both superoxide and nitric oxide play a role in the production of PGE_2. Both of these oxidants can influence the production of PGE_2 by affecting COX-2 activity and therefore play a role in PGE_2 production.

For example, there are studies which suggest that superoxide also plays a role in PGE_2 production. There is evidence that superoxide leaked from the mitochondria has been seen to induce COX activity and PGE_2 production (Blanco et al. 2008). It has been seen that as the mitochondria continues to leak superoxide, and there is increased level of mitochondrial dysfunction within cells, PGE_2 production is altered (Blanco et al., 2008). Interestingly there is also evidence of a feedback loop between superoxide and PGE_2. A recent study demonstrated that PGE_2 can also play a role in mitochondrial dysfunction by altering the membrane potential of the mitochondrial membrane, leading to increased production of ROS, including superoxide (Corrado et al., 2018). Therefore, it is worthwhile to investigate mitochondrial derived forms of ROS, namely superoxide. In a more recent study on ALS, it was observed that ROS levels are altered due to the activation of the EP2 receptor of PGE_2 in motor neurons. (Kosuge et al., 2020). These findings also suggest that PGE_2 does indeed influence the production of ROS, including superoxide.

There are also examples where nitric oxide derived from NOS, can bind to binding sites on the COX-2 enzyme, thus altering the production of PGE_2 (Sangwon, 2014). nNOS and iNOS, have been seen to bind to COX-2, which in turn influences the production of many prostaglandins, including PGE_2 (Arcoleo et al., 1995). Furthermore, there has also been evidence that PGE_2 can influence the production of NO. A recent study indicated that PGE_2 influences the function of iNOS, thus altering the production of NO in murine cells (Cheng et al., 2011). Based on these findings, there is evidence which indicates that there is indeed a feedback loop between nitric oxide and PGE_2.

2.3.3 Hypothesis

I hypothesize that exposure to PGE_2 will induce the production of mitochondrial-derived superoxide anion, and nitric oxide produced from NOS, in a dose-dependent manner, in differentiated neuronal cells.

2.3.4 Methodology used to test the hypothesis

Fluorescence microscopy will be used to detect the fluorescence intensity (FI) of superoxide and nitric oxide production over time of differentiated neuronal cells from four different treatment conditions. Two dyes will be used, MitoSox Red dye, which is specific for labelling superoxide derived from mitochondria, and DAF-2DA, which is specific for labelling nitric oxide found in the cytosol of the cell

2.3.5 Experimental Model System

I will be testing my hypothesis by using neuroectodermal (NE-4C) stem cells, a well-established *in vitro* model system in our lab. The cells were produced from primary brain cell cultures of fore-and midbrain vesicles of 9-day old mice embryos lacking functional p53 protein. In studies previously conducted in our lab, it was determined that NE-4C cells are a suitable model system for experiments regarding PGE_2, due to the fact that NE-4C cells contain all four

EP receptors (Wong et al., 2014). Therefore, any process being investigated can be tested with these cells, since all the receptors which need to be bound to are present (Wong et al., 2014). Furthermore, NE-4C cells undergo differentiation and become neuronal cells, similar to those found in the healthy brain since they undergo proliferation, migration and differentiation, into neurons (Varga et al., 2008). Using differentiated neuronal cells allows for insight into how altered PGE_2 concentrations can affect neurological processes and neurodevelopmental disorders. These cells will undergo differentiation over an 8-day period, using serum deprivation. On the eighth day of differentiation, experiments were conducted. Cells are said to be fully differentiated on Day 8, due to the downregulation and absence of specific genes associated with stem cells and then the presence of genes associated with neuronal cells, being expressed (Wong et al., 2014).

2.3.6 Changes to the thesis content related to COVID-19

As a result of COVID-19 related university and lab closures, some aspects of study 2 could not be completed. For example, performing real-time PCR in PGE_2 treated NE-4C cells to identify the expression level of several ROS regulated genes and NOS isoforms. Additionally, I was not able to complete the additional MitoSox Red assay experiments to obtain more accurate traces for the positive control and some experimental samples. Due to these limitations we added study 1, which could be completed remotely and had some relevance to study 2. Study 1 uses new experimental approaches and shows new skills added to the thesis.

Chapter 3-Methodology

3.1 Study 1: Identification of genes associated with mitochondrial function, inflammatory responses, and synaptic plasticity using whole genome microarray data from males and females COX2-/- offspring

3.1.1 Animal Model

Pregnant females which were heterozygous for the COX-2 gene (B6; 129P2-Ptgs2$^{tm1/unc}$) were obtained from Taconic Bioscience. These mice are known to have a disruption in Ptgs2 gene expression. Female homozygous mice become infertile due to these disruptions, therefore heterozygous COX-2 female mice had to be bred with homozygous knockout males (*COX-2*$^{-/-}$) (Wang et al., 2010). This model has been used in many other papers as a means to investigate the molecular mechanisms of this genetic deficit in COX-2, and to see how it relates to ASD (Liu et al., 2017, Kwan et al., 2016). Furthermore, wild-type mice were also bred to serve as a control. Mice were bred and housed at York University on a 12-hour light/dark cycle and animals were provided with unlimited food and water. In order to determine the embryonic day, a vaginal plug is used to identify Day 1. On days 16 and 19, samples are collected from the *COX-2*$^{-/-}$ and wild-type mice, and they are placed in a TRIzol solution. Three samples from the male and female *COX-2*$^{-/-}$ mice and wild-type mice were collected.

3.1.2 Microarray Analysis

Once samples were collected, total RNA was extracted using the TRIzol method (Fisher Scientific, Toronto, Canada). Following this, RNA samples were taken to the Princess Margaret Genomic Center (Toronto, Canada). Here, whole genome microarray was conducted, data was analyzed, and quality control measures were taken. For male samples, Mouse WG-6 V2 BeadChip (Illumina) microarray was conducted, whereas Mouse Genome 2.0ST GeneChip (Affymetrix) microarray was conducted for females. This is due to the fact that the microarray analysis was conducted on female mouse samples after a period of time where the BeadChip used on the male samples was discontinued. The microarray for both sexes contained 45 281 target probes which came from the National Center for Biotechnology Information Reference Sequence (NCBI) RefSeq (Build 38 Release 22), the Mouse Exonic Evidence Based Oligonucleotide, and the RIKEN FANTOM2 database. Statistics were conducted using R.

3.1.3 Identification of differentially expressed genes

Following the microarray analysis, all genes which were identified as differentially expressed in the *COX-2*$^{-/-}$ mice were normalized to the expression of the same genes found in the WT mice. This comparison was done for genes expressed in the males and females at both E16 and E19. This normalization resulted in a fold change, where the WT mouse gene expression was set as 1. Genes were identified as dysregulated if the fold change of their expression was ≥1.5. Using this cut-off point, genes of interest from three different functional groups; mitochondrial function, inflammatory responses, and synaptic plasticity, were screened and any genes where the expression was ≥1.5, were investigated for this study. Genes from these functional groups were investigated because there is abundant research to connect these

functions and pathways with ASD and other neurological disorders, as seen in many publications in our lab (Rai-Bhogal et al., 2017, Wong et al., 2015, Wong et al., 2018). Genes were identified as being involved in mitochondrial function, inflammatory responses or synaptic plasticity using the software program DAVID Bioinformatics (http://david.abcc.ncifcrf.gov/).

3.1.4 Bioinformatics and analysis of biological function

The gene list generated using DAVID Bioinformatics was then cross-referenced to the microarray list of genes to determine at what stage the gene was dysregulated and among which sex. Following this, using one of the extensions from DAVID Bioinformatics known as Kyto Encyclopedia of Genes and Genome (KEGG), we were able to identify specific functions, pathways, and diseases associated with each gene.

3.1.5 Identification of ASD-risk genes and gene interaction

Once details about each gene was noted, the Autism Database (AutDB, http://autism.mindspec.org/) and KEGG were used in order to identify which genes were ASD-risk genes and publications which provided insight into the gene's link to ASD were recorded. Next, another software program, Cytoscape (http://cytoscape.org) and the plugin GeneMania (http://GeneMania.org) were used to illustrate connections between the genes, based on if the genes played a role in similar pathways. This was done by inputting the list of genes from each functional group into GeneMania separately and identifying the connections. Once interaction plots were developed for each subset of genes, GeneMania was used once more to identify genes which were connected between all the groups. Here, genes from all the groups were inputted and an interaction plot was created highlighting connections between genes involved in the same pathways, but from a different functional group.

3.2 Study 2: The effects of PGE_2 on the production of superoxide and nitric oxide, in differentiated neuronal cells

3.2.1 Cell cultures and Differentiation

As mentioned previously, NE-4C stem cells were used for Study 2. Stem cells were maintained in minimum essential media (MEM). In order for cells to become differentiated, they had to undergo serum deprivation. The cells were differentiated by first plating undifferentiated NE-4C stem cells onto a 60mm plate with Minimum Essential Media (MEM). The next day MEM was removed and neurobasal media supplemented with B-27, was added to the plate (Day 0 of differentiation). This media mixture is a form of serum free media (SFM). Media was changed every other day and by day 6 of differentiation, neurospheres had formed and were floating within the plate. These neurospheres were collected and disrupted in a 15mL centrifuge tube, then they were pipetted back onto their plates, in fresh SFM. On day 8, cells were fully differentiated and experiments were conducted.

3.2.2 Superoxide detection using fluorescence microscopy

Following differentiation (on day 8), fluorescence microscopy was used to detect mitochondrial derived superoxide production within NE-4C cells. Prior to treatment with our four conditions, SFM was removed from the 60mm plates and the cells were then treated with PGE_2, H_2O_2, or left untreated in PBS. Cells were imaged using Mitosox Red which has an emission of 510 nm.

Four different treatment groups were investigated. First the untreated differentiated cells were used as the basal condition. Here, cells were incubated in PBS without additional reagents. Next, there was the1µM or 10µM PGE_2 treated cells. For these treatments, media was removed

and PBS is added to the plate. 1µM PGE_2 or 10µM PGE_2 is then added to the cells and the plate is incubated for 30 minutes. Following this, the plate is washed 3 times with PBS, and then the cells are left in PBS for experiments. Finally, the positive control, 100µM H_2O_2, is used. For this treatment, media is removed and PBS is added to the plate. 100µM H_2O_2 is added to the plate and this is incubated for 1 hour. Following this, the plate is washed 3 times with PBS, and then the cells were left in PBS for experiments.

With this dye, the cells are incubated in 5mM MitoSox Red for 30 minutes before imaging begins. Therefore, the dye is added along with the PGE_2 or, for the plates containing H_2O_2, the dye is added 30 minutes into the H_2O_2 incubation, so the cells are exposed to the dye for just 30 minutes. Following these incubations, the plate is imaged using the fluorescence filter TRITC, for two hours. Images are taken every 30 seconds, so there was a total of 240 images per condition.

After the cells were imaged, the amount of fluorescence was quantified using the NIS element software. This was done by selecting 20 cells per plate for each biological replicate. Then, the software was used to quantify the intensity of fluorescence generated over a two-hour period. This was used to indicate how the concentration of superoxide produced changed throughout the experiment.

Following this, another analysis was conducted in order to calculate the kinetic rate of superoxide production for each treatment. This was done by first identifying the point of inflection of each plot generated from the intensity of fluorescence over time (plot used for concentration analysis). After the exponential growth of each plot was found, the maximum slope was found. This maximum slope is the kinetic rate of each plot. The kinetic rate is a quantity which is used to indicate a ratio of how much superoxide is being generated per minute.

The rate was found for each of the three replicates of each treatment, which were then averaged, in order to give an average rate for each treatment. Therefore, there were 3 kinetic rates for each treatment which were averaged to give one representative rate for the respective treatment.

3.2.3 Nitric oxide detection using fluorescence microscopy

For the nitric oxide detection, similar procedures were followed as for the superoxide detection. Cells were grown and differentiated in the same manner. In order to quantify the level of intracellular nitric oxide in NE-4C cells, the fluorescent dye of 4, 5 diaminofluroescein diacetate (DAF-2 DA) was used, which has an emission of 515 nm. Images were taken using the FITC filter.

Four different treatment groups were investigated. First the untreated differentiated cells represented the basal condition. Cells were incubated in PBS without additional reagents. Next, there were the 1µM PGE_2 and 10µM PGE_2 treatments. For these conditions media was removed and PBS is added to the plate. 1µM PGE_2 or 10µM PGE_2 is then added to the cells and the plate is incubated for 30 minutes. Following this, the plate is washed 3 times with PBS, and then the cells are left in PBS for experiments. Finally, the positive treatment, 1 µg/mL LPS is used. Here, media is removed and PBS is added to the plate. 1 µg/mL LPS is added to the plate and this is incubated for 2 hours. Following this, the plate is washed 3 times with PBS, and then the cells are left in PBS for experiments.

Afterwards, 5 µM of DAF-2DA was added to the cells and they were imaged for 45 minutes. This means that the dye was not incubated prior to imaging. An image was taken every 30 seconds, using a time-lapse florescence microscope system (Nikon Eclipse Ti-E) with NIS Elements software.

Following this, the same type of analysis was conducted as with the superoxide detection. First, concentrations were analysed using DAF2-DA and fluorescence microscopy. Then kinetic rates were calculated by finding the point of inflection for each treatment.

3.3 Statistical analysis

3.3.1 Statistical analysis conducted for superoxide detection in differentiated NE-4C cells

Once the fluorescence data was quantified from the trials using MitoSox Red, further statistical analysis was conducted. First, statistics were conducted on the concentration data collected. Using SPSS, randomized block analysis was conducted using the maximum point of fluorescence per cell, per condition. For example, fluorescence data was collected for 80 cells per condition. Then the maximum point of fluorescence was taken from each of the 80 cells, and a randomized block test was then conducted. Also a Tukey's post hoc test was done, with an alpha of 0.05, to indicate any significant differences between the different treatments. These statistics were conducted to see if there is a significant difference in the concentration of superoxide produced between each treatment.

Next, statistics were conducted for the kinetic rates of each treatment. This was done using an unpaired two-sample T-test. T-tests were conducted using SPSS, from the kinetic rates calculated for each of the three replicates calculated for each of the treatments. Therefore, for each treatment n=3, and the kinetic rates from each replicate were averaged to give the average kinetic rate for each treatment. Significance was determined if the p-value was said to be less than 0.05.

3.3.2 Statistical analysis conducted for nitric oxide detection in differentiated NE-4C cells

Statistics conducted for nitric oxide are done in the same manner as for the superoxide detection. However, data was collected from 15 cells per each plate. Therefore, there were 60 cells analysed for each of the 4 treatments.

Then, a randomized block design was conducted, followed by a Tukey's post hoc. This was done on the concentration data, in order to determine if there is a significant difference in the amount of nitric oxide produced between the treatments.

Once the concentration data was analysed, kinetic rates were analysed. This was also done using a two-sample T-test, which averaged the 3 kinetic rates determined for each treatment. Significance was determined if the p-value was less than 0.05.

Chapter 4- results

4.1- Study 1: Identification of genes associated with mitochondrial function, inflammatory responses and synaptic plasticity, using whole genome microarray data from males and females COX2-/- offspring

4.1.1 Identification of genes from the microarray analysis, associated with mitochondrial function, inflammatory response, and synaptic plasticity

A mouse genome microarray was conducted on brain tissue collected at embryonic day 16 (E16) and embryonic day 19 (E19), from *COX-2*$^{-/-}$ knockout mice and wild-type (WT) mice. The microarray analysis was conducted previously in our lab, where gene expression from both male and female *COX-2*$^{-/-}$ knockout mice, which were expressed at E16 and E19, were determined and compared to the gene expression of WT male and female mice (Rai-Bhogal et al., 2017). Following this analysis, the expression of the genes was noted as a fold change compared to the WT expression. Differentially expressed genes with fold change values ≥ 1.5 were scrutinized and further investigated.

By using the microarray data, insight can be gathered about how a PGE_2 deficiency can lead to differentially expressed genes associated with mitochondrial function, inflammatory responses, and synaptic plasticity. There has been an abundance of evidence in recent years, connecting abnormalities in PGE_2 and ASD (Tamiji and Crawford, 2010, Dean et al., 2012, Wong et al., 2017, Rai-Bhogal, 2017). Furthermore, genes associated with these three functions are of interest because many studies have linked abnormalities in mitochondrial function, inflammatory responses and synaptic plasticity with ASD. Additionally, sex is of interest because many neurodevelopmental disorders, including ASD, present a sex-bias where males are more likely than females to have the disorder.

Using the data collected from the microarray analysis, several differentially expressed genes involved in multiple pathways were identified based on if their fold change values were ≥ 1.5. The genes were of interested if they are involved in mitochondrial function, inflammatory responses, or synaptic plasticity. As seen in Table 1, 12 genes were identified as differentially expressed in the *COX-2*$^{-/-}$ knockout mice. In Figure 4, the localization of these genes in neuronal cells is illustrated

Each of the12 genes is differentially expressed in either males or females, but never in both sexes. For example, in Table 1, it is observed that *Ndufa3, Ndufc2, ATP5e, Sdhc, Eif3c, EiF4a2, and GRiA2* (7 genes) are differentially expressed in only females while the genes, *Ndufb10, TSC2, NFiB, TGFRB2 and MEFC2* (5 genes) are differentially expressed in males only. None of the 12 genes were dysregulated in both males and females. This indicates that sex may be a key factor in determining which genes become differentially expressed since the genes are specifically expressed between either of the two sexes.

Furthermore, most of the 12 genes identified are differentially expressed at only one stage. As seen in Table 1, *Ndufa3, TGFRB2, MEFC2, GRiA2, Sdhc, EiF3c, and NFiB* (7 genes) were dysregulated at E16. However, only *Ndufc2 and ATP5e* (2 genes), were dysregulated at E19. Interestingly, *Ndufb10, EiF4a2, and TSC2* (3 genes) were dysregulated at both E16 and E19. This is interesting because it suggests that certain genes may become differentially expressed due to abnormalities during specific stages of development, since a majority of the genes in Table 1 are differentially expressed in only 1 specific stage.

All genes listed in Table 1 are either upregulated or downregulated. Upregulated values are seen in green, whereas downregulated gene expression in seen in red. As mentioned previously, 9 genes are seen to be dysregulated at one specific developmental stage, whereas 3

genes were observed to be dysregulated at both E16 and E19. A majority of the genes dysregulated at only one stage, were observed to be upregulated compared to the WT mice. 7 genes (*Ndufa3, Ndufc2, ATP5e, Sdhc, EiF3c, NFiB, and TGFRB2*) were upregulated at one stage and 2 genes (*MEFC2 and GRiA2*) were downregulated. Of the 3 genes which were dysregulated at both E16 and E19, it was seen that the genes were dysregulated differently at each stage. For instance, 2 of the genes (*EiF4a2 and TSC2*) showed a downregulation at E16, but an upregulation at E19. However only 1 gene (*Ndufb10*) showed an upregulation at E16, which shifted to a downregulation at E19.

Furthermore, out of all the genes, *Ndufa3, Sdhc, and EiF3c,* were upregulated in females at E16, while *Ndufc2 and ATP5e* were upregulated at E19 in the females. Also, *GRiA2* is downregulated at E16 for females, and there are no genes downregulated for females at E19. These trends do not include *EiF4a2* which is dysregulated in females at both stages, exhibiting a downregulation at E16 and an upregulation at E19. As for males, *NFiB and TGFRB2* are upregulated at E16 while no genes are upregulated at E19. There are no downregulated genes for males. Again, these trends do not include the 2 gene which are dysregulated in males at both E16 and E19. *Ndufb10* is upregulated at E16 and downregulated at E19, whereas *TSC2* is downregulated at E16 and upregulated at E19.

In figure 4, the localization of the 12 genes gathered from the microarray analysis is depicted. It is observed that a majority of the genes gathered from the microarray data are localized within the mitochondria of neuronal cells. These genes include *Ndufa3, Ndufc2, Ndufb10, ATP5e,* which are a part of the electron transport chain (ETC), and *Sdhc, EiF3c, EiF4a2, and TSC2*, which can be found in the mitochondrial matrix. These 8 genes play crucial roles in correct mitochondrial function, but also have roles in some other biological processes,

which will be highlighted in Table 3 and Figure 8. The other 4 genes gathered from the microarray data, *NFiB, TGFRB2, MEF2C, and GRiA2*, can be localized in the cytosol of neuronal cells and play roles in many other pathways, such as those involving inflammatory responses and synaptic plasticity. This will also be discussed further.

The above observations are interesting since they demonstrate many of these gene dysregulations are sex-specific and only occur at one stage. A majority of the genes that were differentially expressed at E16 returned to a normal level by E19 to show similar gene expression as the WT mice. This may suggest that there are possible compensatory mechanisms which the developing mouse may undergo as a means to combat the dysregulations that the *COX-2*$^{-/-}$ knockout mice undergoes. A compensatory mechanism would allow the organism to normalize the expression of a dysregulated gene, in order to reduce the risk associated with prolonged abnormal gene expression. This could explain why there are different expressions of the genes at different stages of development. This can especially be suggested for the 3 genes which are differentially expressed at both stages. These genes have a shift in their expression from one stage to the next, which may be due to an overcompensation for the abnormal expression at E16, which can lead to an opposite dysregulation at E19. However, another explanation could be that normally, a specific gene is only required at a critical period, which would also lead to shifts in gene expression between stages.

Furthermore, the genes listed in Table 1 have been associated with many key functions that are important for prenatal brain development and are also associated with ASD. These genes can be divided into three main functional groups which are, mitochondrial function (*Ndufa2, Ndufb1, Nduf3c, ATP5e, Sdhc, EiF3c, EiF4a2, TSC2*), inflammatory responses (*NFiB,*

TGFBR2) and synaptic plasticity (*MEFC2, GRiA2*). Some characteristics of these genes and their interactions to other genes, will be discussed further.

Table 1. Fold change of COX-2$^{-/-}$ differentially expressed genes obtained from microarray analysis for male and female mice at E16 and E19. The fold change expression of differentially expressed genes found in male and female COX-2$^{-/-}$ mice at embryonic day 16 (E16) and embryonic day 19 (E19). A green number indicates and upregulation, whereas a red value indicates a downregulation. Fold changes were obtained by comparing genes expression to a Wild-type mouse model. Whole genome microarray analysis was conducted to determine the expression of the genes. A negative value indicates a downregulation whereas a positive value indicates an upregulation. The functional group which each gene will be categorized under is also noted. This group is determined by the primary role these genes play in neuronal cells.

Gene	Gene Name	Functional Group	Female E16	Male E16	Female E19	Male E19
Ndufa3	NADH: ubiquinone oxidoreductase subunit A3	Mitochondrial	+1.53	-	-	-
Ndufc2	NADH: ubiquinone oxidoreductase subunit C2	Mitochondrial	-	-	+1.52	-
Ndufb10	NADH: ubiquinone oxidoreductase subunit B10	Mitochondrial	-	+1.78	-	-6.53
ATP5e	ATP Synthase Epsilon Chain E	Mitochondrial	-	-	+1.82	-
Sdhc	Succinate Dehydrogenase Complex Subunit C	Mitochondrial	+1.68	-	-	-
Eif3c	Eukaryotic Translation Initiation Factor 3 Subunit C	Mitochondrial	+1.88	-	-	-
Eif4a2	Eukaryotic Translation Initiation Factor 4 Subunit A2	Mitochondrial	-1.91	-	+1.59	-
TSC2	Tuberous Sclerosis 2 Protein	Mitochondrial	-	-2.07	-	+1.83
NFiB	Nuclear Factor IB- Type	Inflammatory	-	+1.612	-	-

TGFRB2	Transforming Growth Factor B Receptor 2	Inflammatory	-	+1.54	-	-
MEF2C	Myocyte Enhancer Factor 2C	Synaptic Plasticity	-	-2.96	-	-
GRiA2	Glutamate Receptor IonotropicAMPA Type 2	Synaptic Plasticity	-3.14	-	-	-

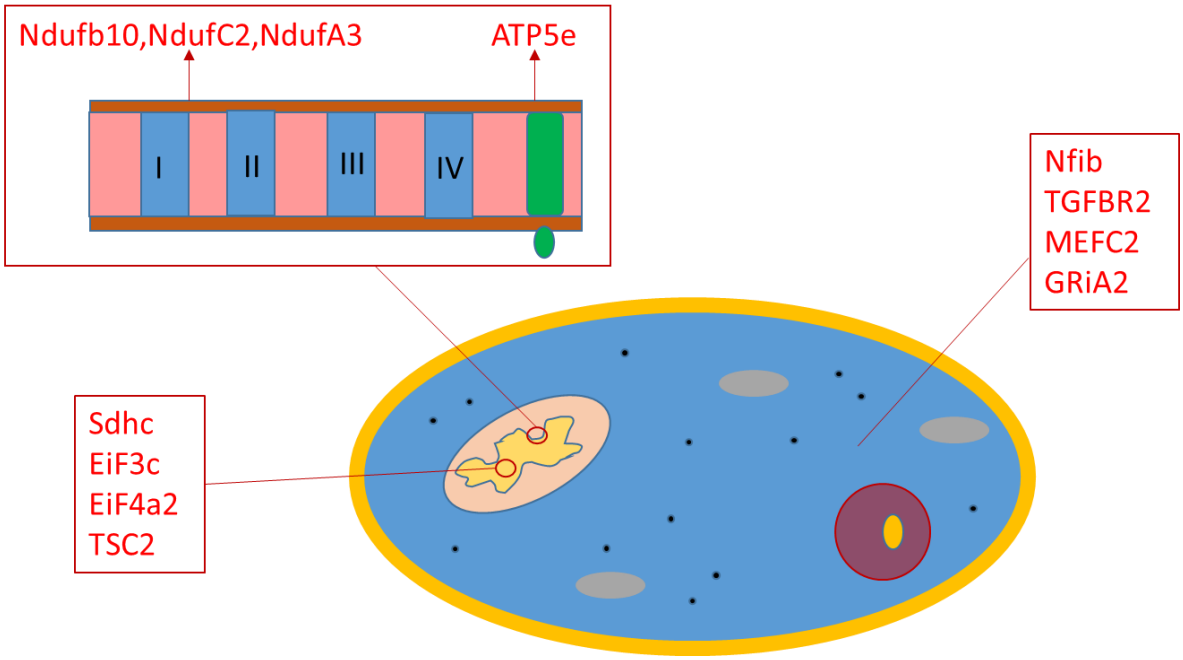


Figure 4. Localization of genes associated with mitochondrial function, inflammatory response, and synaptic plasticity based on interactions determined using Cytoscape software. A list of genes associated with mitochondrial function, inflammatory response, and synaptic plasticity was obtained using Cytoscape. The localization of the genes was determined using DAVID bioinformatics software. Genes were either expressed in the mitochondrial matrix, electron transport change, or cell cytosol. Genes expressed in the electron transport chain are expressed in one of the five complexes (I-IV and green channel). The mitochondrial genes of interest were primarily expressed in complex I and ATP Synthase (green channel).

4.1.2: Analysis of mitochondrial gene interaction and localization, using interaction plots generated with Cytoscape, for mitochondrial genes dysregulated in the microarray analysis of COX-2$^{-/-}$ knockout mice

Mitochondrial dysfunction plays a role in the brain and has been associate with several neurological disorders, including ASD. For instance, many symptoms associated with increased levels of mitochondrial dysfunction, such as intellectual disability, language and speech impairments, and behavioural abnormalities, have also been associated with disorders such as, ASD, Alzheimer's disorder, Parkinson's disorder, and Schizophrenia (Guilivie et al., 2010, Goh et al., 2014). One manner in which mitochondrial dysfunction can occur is via gene dysregulation (Goh et al., 2019). Therefore, we have investigated how a COX2-PGE_2 signalling deficit can influence genes associated with mitochondrial function.

Once genes from the *COX-2*$^{-/-}$ knockout mice microarray analysis were identified to be associated with mitochondrial function using various literature and DAVID Bioinformatics (https://david.ncifcrf.gov), they were analyzed using the Cytoscape GeneMania plugin (GeneMania.org). This plugin was used to illustrate the interactions of the 8 mitochondrial genes found from the microarray analysis. These 8 genes are *Ndufa3, Ndufc2, Ndufb10, ATP5e, Sdhc, EiF3c, EiF4a2 and TSC2*. The interactions between the genes were determined by the software based on similarities in the pathways and biological processes the genes are involved in. These interactions are illustrated in Figure 5. Following the use of Cytoscape, the genes were analysed using DAVID Bioinformatics once more, to determine the localization of the genes within the mitochondria of neuronal cells. This is also shown in Figure 5.

In Figure 5, it is observed that there are 8 genes, *Ndufa3, Ndufc2, Ndufb10, ATP5e, Sdhc, EiF3c, EiF4a2 and TSC2*, which were dysregulated in the microarray analysis of the *COX-2*$^{-/-}$

knockout mice, which are also associated with mitochondrial function. In Figure 5, differential gene expression in males is depicted by blue triangles, whereas red triangles indicate female dysregulations. Upward facing triangles are representative of upregulations of the specific gene and downward facing triangles represent downregulations. All expressions are compared to a wild-type mouse model. Differential gene expression at E16 is shown on the left of the figure and differential expression at E19 is shown on the right. ASD-risk genes are identified by an asterisk. The localization of the gene expression is shown by rectangles indicating if the genes are primarily involved in the mitochondrial membrane potential and the electron transport chain (ETC), ATP synthesis, or as a secondary mitochondrial enzyme.

Using this figure, it is observed that *Ndufb10, and TSC2* are dysregulated in males, whereas *Ndufa3, Ndufc2, ATP5e, Sdhc, EiF3c, and EiF4a2* are dysregulated in females, among the two embryonic stages. This is noteworthy because this could indicate a sex-bias in terms of differential gene expression.

In terms of differential gene expression, there are many interesting observations which can be made between E16 and E19. At E16, 6 out of the 8 mitochondrial genes are seen to be differentially expressed. *Ndufa3 and Ndufb10* are directly involved in the electron transport chain, whereas *Sdhc, EiF3c, EiF4a2, and TSC2*, are all secondary enzymes which act upon the mitochondria. Interestingly, it was observed that *Ndufa3, Sdhc, EiF3c, and Eif4a2* were dysregulated in females, whereas *Ndufb10 and TSC2*, were dysregulated in males. Among these genes at E16, *Ndufa3, Sdhc, EiF3c, Eif4a2, and TSC2* are downregulated and *Ndufb10* is upregulated. Interestingly, all the female genes were seen to be upregulated while for the male genes *Ndufb10* was upregulated and *TSC2* was downregulated. Therefore, there is just one downregulated gene observed at E16, which is found in males.

Furthermore, as mentioned previously, all of the genes found in the microarray data were divided into subsets of genes based on function. The mitochondrial subset set of genes are the only set of genes which demonstrate shifts in the expression of certain genes at different stages. At E19, there are also only 6 out of 8 mitochondrial genes that are differentially expressed, but a few of them are different from those differentially expressed in E16. Here, *Ndufc2, Ndufb10 and ATP5e* are all involved in the ETC, with *ATPE5e* being specifically involved in the ATP synthesis aspect of the ETC. However, *EiF4a2 and TSC2* are involved in the mitochondria as secondary enzymes. Furthermore, there is a shift at E19, where it is observed that *Ndufc2 and ATP5e* are now dysregulated, but they were not at E16, and *Sdhc and Ndufa3* are no longer dysregulated at E19. Additionally, there are shifts in the dysregulation pattern of *Ndufb10, TSC2, and Eif4a2*, which will be discussed further.

In terms of sex differences, *Ndufc2, ATP5e and EiF4a2* are differentially expressed in females, while *Ndufb10 and TSC2* are differentially expressed in males at E19. Of these genes, those dysregulated in females are all upregulated, whereas for the genes associated with males, *Ndufb10* is downregulated and *TSC2* is upregulated. Therefore, there is only one downregulated gene at E19 that is observed on in males.

There are 3 genes associated with mitochondrial function which demonstrate a change in regulation pattern at E16 and E19. For example, *TSC2 and EIF4a2* are downregulated, while *Ndufb10* is upregulated, at E16. However, at E19 the opposite is observed where *TSC2 and EIF4a2* are upregulated and *Ndufb10* is downregulated at E19. As mentioned previously, this shift in dysregulation can be due to an overcompensation during development, in an attempt for the *COX-2*$^{-/-}$ knockout mice to normalize the dysregulation seen at E16.

In Figure 5, the interactions between each gene are also illustrated. It can be observed that the genes which are primarily involved in the electron transport chain (ETC) are all connected to each other. This is due to the fact that these genes interact with one another to help transport electrons through the various complexes of the ETC. These genes are also connected to genes outside of the ETC. Each of the ubiquinone genes which act upon the ETC also interact with at least one other gene from outside the ETC. Also, *Ndufb10 and Ndufa3* are connected to ATP5e, but *Ndufc2* is not. This suggest that *Ndufb10 and Ndufa3* interact with genes further down the ETC than *Ndufc2*. Also, since each ubiquinone gene connects to genes outside the ETC, this indicates that genes from the ETC also interact with genes in the mitochondrial matrix. This is interesting because this means that genes from the ETC can influence genes in the matrix, which are also known to be connected to genes outside of the mitochondria, which can potentially influence many other cellular processes and pathways. Therefore, the ETC complex genes may not just be associated with the mitochondria. The localization of each gene is demonstrated in Figure 5. This figure illustrates that the 8 genes associated with mitochondrial function are localized in very distinct regions of the mitochondria. However, by comparing this figure to Figure 4, it can be observed that the genes localized to separate areas of the mitochondria, interact with genes in other parts of the mitochondria as well. Also, most of the genes which are associated with secondary enzymes outside of the ETC are connected to one other secondary enzyme gene and one gene localized in the ETC. However, *EiF4a2* is the exception to this rule, as it is connected to 2 secondary genes and no genes found within the ETC.

Furthermore, in Figure 5, ASD-risk genes are identified with an asterisk. There are 4 ASD-risk genes, *ATP5e, TSC2, Sdhc, and EiF3c,* which are associated with mitochondrial

function. Of the 4 risk genes, *ATP5e, TSC2, and Sdhc* are connected to one another. This may suggest that these 3 genes may directly influence each other and dysregulations in one of these genes may result in dysregulations in the other two, which can lead to ASD. However, although *EiF3c* is not directly connected to the other ASD-risk genes, it may be indirectly connected to the other 3. For instance, EiF3c is connected to *EiF4a2*, which is connected to Sdhc, which could be the possible link between EiF3c and the other 3 ASD-risk genes.

Additionally, these 4 ASD-risk genes also show interesting dysregulations between E16 and E19 and both sexes. For instance, at E16 3 of the 4 ASD-risk genes are seen to be dysregulated with 2 of them being upregulated. At E19, 2 ASD-risk genes are differentially expressed, both of which are upregulated. *Sdhc, EiF3c and ATP5e* are all upregulated in females either at E16 or E19. However, *TSC2* is downregulated at E16 and upregulated at E19, in males. These are fascinating observations because these trends can indicate that the sex bias associated with these genes can be linked to the sex difference observed with ASD. Finally, since there is a shift in the dysregulation of *TSC2* between both stages, *TSC2* may contribute to ASD in multiple ways, since the shift in dysregulation can alter pathways which the gene is involved in, at E16 and then at E19, in multiple ways that can lead to ASD. This indicates that *TSC2* is a gene which must be carefully regulated because any dysregulation can have severe effects.

Therefore, interesting trends were observed among the differentially expressed genes associated with mitochondrial dysfunction. For starters, since all the genes in this category were found to be differentially expressed in the microarray data, this may indicate that the COX2-PGE_2 signalling pathway plays a crucial role in mitochondrial function. Furthermore, seeing as there were more genes differentially expressed in females, this can indicate a sex bias in the differential gene expression among genes associated with mitochondrial function.

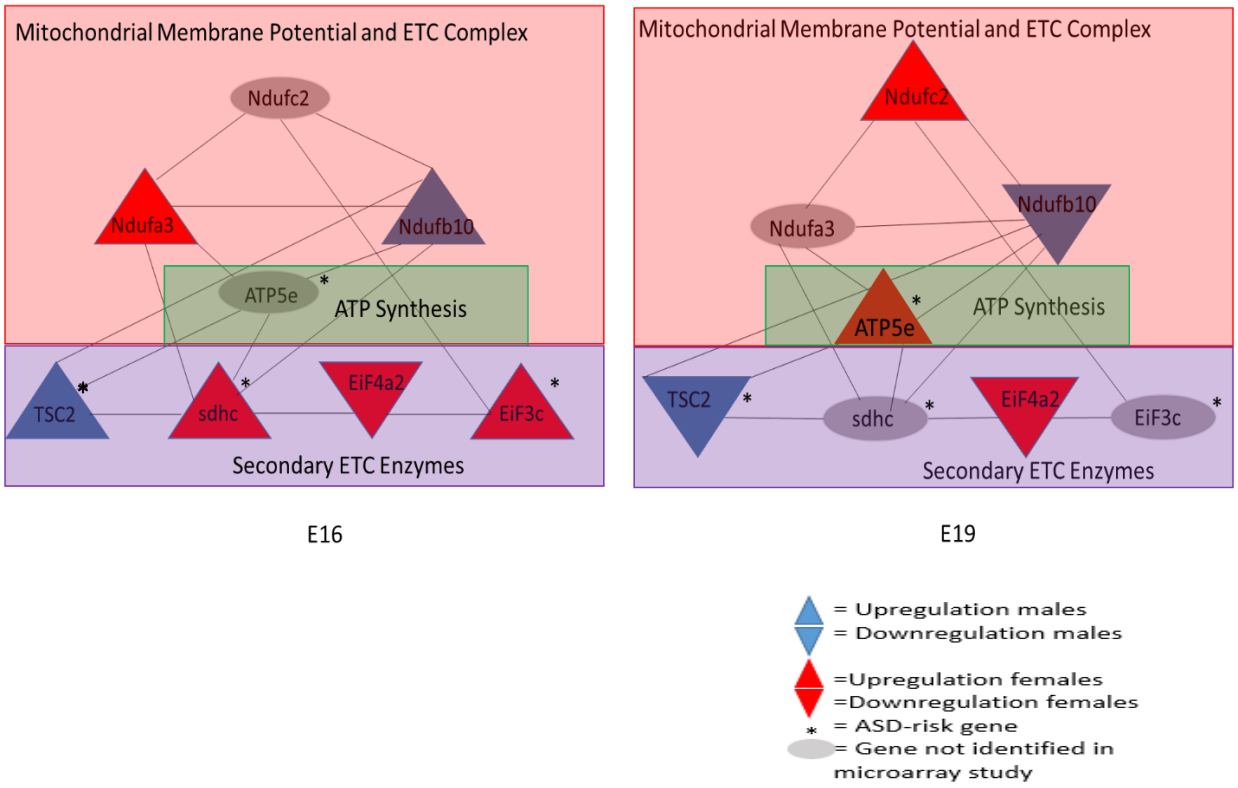


Figure 5. Comparison and localization of differential gene expression for genes associated with mitochondrial function from the microarray analysis of COX-2$^{-/-}$ mice at E16 and E19. The expression of genes associated with mitochondrial function were determined using data from the microarray analysis of COX-2$^{-/-}$ male and female mice. Differential gene expression in males is depicted by blue triangles, whereas red triangles indicate female dysregulations. Upward facing triangles are representative of upregulations of the specific gene and downward facing triangles represent downregulations. Differential gene expression at Embryonic day 16 (E16) is shown on the left of the figure and differential expression at embryonic day 19 (E19) is shown on the right. ASD-risk genes are identified by an asterisk. The localization of the gene expression is shown by rectangles. Localization was determined using DAVID bioinformatics. The red rectangle outlines genes involved with the electron transport chain (ETC) and mitochondrial membrane potential. The green rectangle shows genes which play a role in ATP synthesis. The blue rectangle highlights genes which are secondary enzymes found in the mitochondrial matrix that act upon the ETC. Lines are used to connect genes which play a role in similar pathways. These interactions were generated using GeneMania.

4.1.3- Analysis of the interaction of genes associated with inflammatory responses, using interaction plots generated with Cytoscape, for *COX-2*$^{-/-}$ knockout mice

Literature has shown many connections between ASD and abnormal inflammatory responses. Many aspects of abnormal immune response, such as irregular cytokine and microglial activation, are also observed in individuals with neurological disorders like ASD and Alzheimer's disorder (Elias et al., 2015). Inflammatory responses can be altered due to abnormal gene expression, therefore it is worthwhile to investigate if abnormal COX2-PGE_2 signalling can result in the differential gene expression of inflammatory response genes.

Following the *COX-2*$^{-/-}$ knockout mice microarray analysis, 2 genes were identified to be associated with inflammatory responses (*NFiB and TGFRB2)*, using various literature and DAVID Bioinformatics (https://david.ncifcrf.gov). Both of these genes were found to be upregulated at E16, but normally regulated at E19, compared to the WT mice. Genes were then analyzed using the Cytoscape GeneMania plugin (GeneMania.org). This plugin was used to find other genes which are associated with similar processes and pathways as the inflammatory response genes found from the microarray analysis. The interactions were determined by the software based on the similar pathways and biological processes the genes are involved in. These interactions are illustrated in Figure 6.

In Figure 6, it can be observed that there were no genes dysregulated in females however, the two genes dysregulated in males are identified by blue triangles. Upward facing triangles symbolize an upregulation of the gene expression, whereas downward facing triangles represent a downregulation. There were no downregulated genes to report. Differential gene expression was determined by comparing expression from COX-2$^{-/-}$ animals and wild-type animals. Grey

ovals indicate that the gene was not listed as differentially expressed from the microarray analysis, but using GeneMania, a connection between genes in ovals and the genes we found in the microarray data, was observed. Genes listed were not found to be differentially expressed at E19 in the microarray analysis. ASD-risk genes are identified with an asterisk. Lines connecting genes indicate that both genes play roles in similar pathways.

From the microarray analysis, 2 genes were found to be associated with inflammatory responses, *NFiB and TGFRB2*. Using Cytoscape, 6 other genes, *TSC1, PrkcB, NFiX, Acvr1, TGFBR1 and Trp53*, were identified to be connected with the 2 genes from the microarray data, which are also involved in inflammatory responses. This association was determined based on if the genes connected are involved in pathways involved in other biological responses. *TGFBR2 and NFiB* were identified using the microarray analysis to be upregulated in males at E16. These genes are upregulated by a fold change of 1.61 and 1.54, respectively. However, at E19, these genes are no longer differentially expressed. This indicates that there may be a shift in which genes are functionally needed at each specific stage, thus altering the gene expression between E16 and E19. Alternatively, it is also possible that there is a compensatory mechanism which occurs after E16 to allow both these genes to adjust their regulation back to similar values as the WT mice.

In Figure 6, it is evident that many of the genes are interconnected due to connections being highlighted between each gene and multiple others, all of which are involved in similar aspects of the inflammatory response. For example, *Trp53, TGFBR1, and Acrv1* are connected to 4 other genes, whereas genes like *NFiB and TGFBR2* are connected to 7 and 6 genes respectively. *TSC1* has connections to *PrKcB, TGFBR1, Trp53, NFiB, and TGFBR2*. *PrKcB* has connections to *TSC1, Acrv1, TGFBR1, and Trp53*. *Acrv1* is connected to *PrKcB, NFiB, and*

TGFBR2. *TGFBR1* is connected to *TSC1, PrKcB, NFiB, and TGFBR1*. *Trp53* is connected to *PrKcB, TSC1, NFiB and TGFBR1*. *TGFBR2* is connected to all the genes except *NFiX*, whereas *NFiB* is connected to all of the other 6 genes. This illustrates that all of the genes listed in Figure 6 are connected to several other genes which are involved in inflammatory responses, indicating that these genes can play multiple roles in pathways involved in inflammatory responses. The exception is *NFiX*, which is only connected to *NFiB*. This suggests that *NFiB and NFix* work in a very specific manner to contribute to inflammatory responses. Due to the fact that a majority of the genes involved in the inflammatory responses are connected to each other, this could suggest that these genes contribute to similar processes and they could possibly influence the expression of each other along specific pathways.

Although only *TGFBR2 and NFiB* were observed to be differentially expressed in the *COX-2*$^{-/-}$ knockout mice microarray, 5 of the 8 genes depicted in Figure 6 are ASD-risk genes, including the 2 from the microarray data. These genes are *TGFBR2, NFiB, TSC1, PrKcB*, and *TGFBR1*. Interestingly, each of these ASD-risk genes interact with each other. This can be an indication that these genes may influence one another directly and may result in ASD. This means that there is the possibility that a dysregulation in one gene, can lead to dysregulations in others, leading to a higher risk of ASD. For example, *TGFBR2 and NFiB* are associated with upregulations in males at an early stage. This may lead to changes in the inflammatory pathway which can alter the regulation of the other 3 ASD-risk genes. It is also worth noting that the dysregulations seen in *TGFBR2 and NFiB* were only observed in males. This is important because it is known that ASD has a higher prevalence in males, so these observations made from the microarray data could support this.

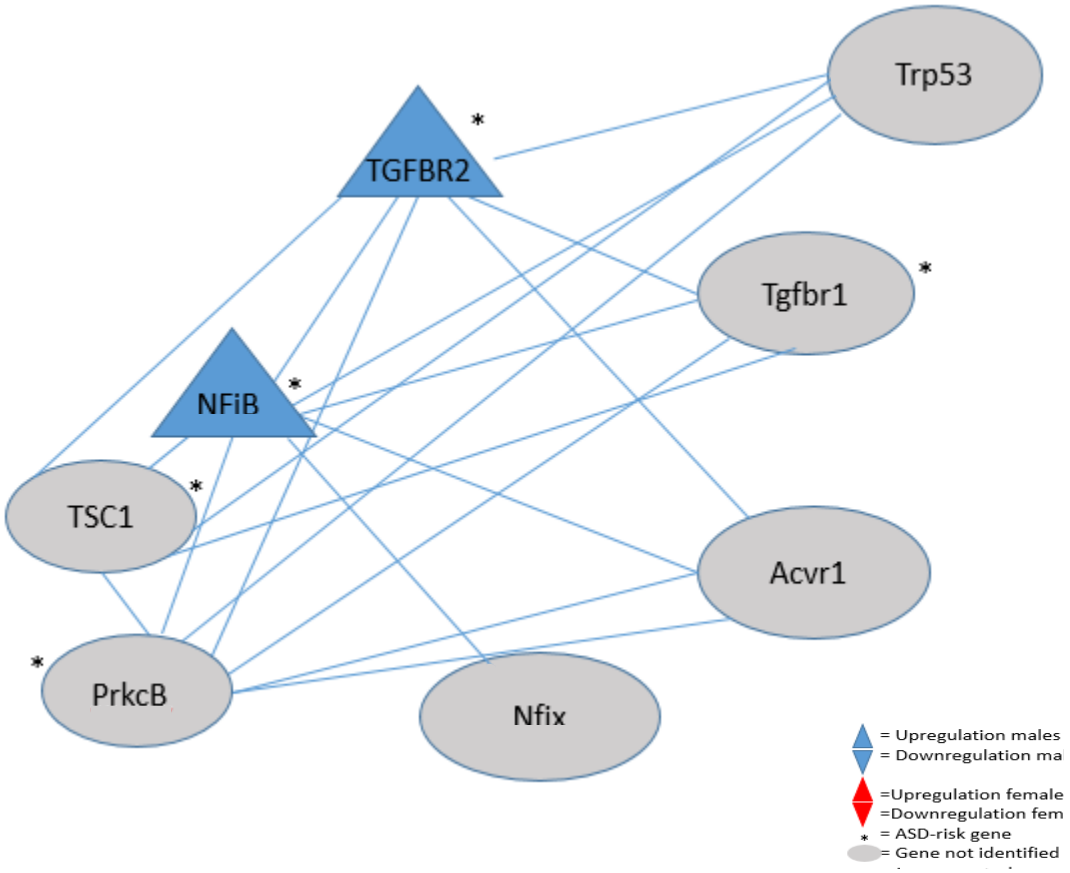


Figure 6. Interaction of gene expression for genes associated with inflammatory responses based on differential gene expression determined from the microarray analysis of COX-2$^{-/-}$ mice at E16. The genes found in the whole genome microarray study for the COX-2$^{-/-}$ model, which were linked to various inflammatory responses at E16, were used to create interaction networks, using GeneMania. Genes dysregulated in females are identified with a red triangle and genes dysregulated in males are identified by blue triangles. Upward facing triangles symbolize an upregulation of the gene expression, whereas downward facing triangles represent a downregulation. Differential gene expression was determined by comparing expression from COX-2$^{-/-}$ animals and wild-type animals. Grey ovals indicate that the gene was not listed as differentially expressed from the microarray analysis. Genes listed were not found to be differentially expressed at E19 in the microarray analysis. ASD-risk genes are identified with an asterisk. Lines connecting genes indicate that both genes play roles in similar pathways.

4.1.4- Analysis of the interaction of genes associated with synaptic plasticity, using interaction plots generated with Cytoscape, for COX-2-/- knockout mice

There has been a lot of research connecting ASD and synaptic plasticity. There has been ample evidence to indicate that there are abnormalities in synaptic plasticity in individuals with ASD (Kelleher and Bear, 2005). Many genes can influence synaptic connectivity and the degree of synaptic plasticity which occurs in the brain. Therefore, investigating if a COX2-PGE_2 signalling deficit can alter the expression of genes associated with synaptic plasticity, could provide insight into how abnormal PGE_2 signalling can contribute to ASD.

Following the *COX-2*$^{-/-}$ knockout mice microarray analysis, genes were identified to be associated with synaptic plasticity using various literature and DAVID Bioinformatics (https://david.ncifcrf.gov). The two genes identified using the microarray analysis were *MEFC2 and GRiA2*. These genes were then analyzed using the Cytoscape GeneMania plugin (GeneMania.org). This plugin was used to find other genes which are associated with similar processes and pathways as the synaptic plasticity genes found from the microarray analysis. The interactions were determined by the software based on similarities in the pathways and biological processes the genes are involved in. These interactions are illustrated in Figure 7.

In Figure 7, it can be observed that genes dysregulated in females are identified with a red triangle and genes dysregulated in males are identified by blue triangles. Upward facing triangles symbolize an upregulation of the gene expression, whereas downward facing triangles represent a downregulation. Grey ovals indicate that the gene was not listed as differentially expressed from the microarray analysis, but through the use of GeneMania a connection was found between the genes in the ovals and genes from the microarray analysis, based on their role in synaptic plasticity. Genes listed were not found to be differentially expressed at E19 in the

microarray analysis. ASD-risk genes are identified with an asterisk. Lines connecting genes indicate that both genes play roles in similar pathways.

From the microarray analysis 2 genes, *MEFC2 and GRiA2*, were found to be associated with synaptic plasticity. Using Cytoscape, 4 other genes, *FmR1, Grin2b, cyfip1 and EiF4e*, were identified to be associated with *MEFC2 and GRiA2*, which are also involved in synaptic plasticity. *MEFC2* was identified using the microarray analysis to be downregulated in males at E16, whereas *GRiA2* was identified to be downregulated in females at E16. The fold change for these genes were 2.96 and 3.14, respectively. However, at E19, these genes are no longer differentially expressed compared to the WT mice. This indicates that there is a possible compensatory mechanism which occurs after E16 to allow both these genes to adjust their regulation back to similar values as the WT mice.

From Figure 7, it can be observed that *GRiA2* is connected to most of the other genes except for *EiF4e*. This means that *GRiA2* is involved in many pathways and its regulation can also influence other genes. *FmR1 and Grin2b* are connected to 3 other genes, whereas as *Cyfip1 and MEFC2* are connected to just 2 other genes. *GRiA2* is connected to *MEFC2, Grin2b, FmR1, and cyfip1*. *MEFC2* is connected to *GRiA2 and Grin2b*. *FmR1* is connected to *GRiA2, Grin2b,* and *cyfip1*. *Grin2b* is connected to *MEFC2, FmR1 and GRiA2*. *Cyfip1* is connected to *GRiA2, FmR1 and EiF4e*. Interestingly, *EiF4e* is only connected to *Cyfip1*, suggesting that it is involved in a very specific manner to synaptic plasticity. These interactions between the genes provide insight into the potential mechanisms the interconnected genes may be a part of, which can influence synaptic plasticity within neuronal cells.

Furthermore, it is important to note that all of the genes associated with synaptic plasticity are also ASD-risk genes. This further points to ASD being associated with

abnormalities in synaptic plasticity and even learning and memory deficits. Also, since *MEFC2 and GRiA2* were determined to be downregulated (fold change of 2.96 and 3.14, respectively) at E16 and not at E19, this indicate that the downregulation at E16 could have lasting detrimental effects in terms of neurogenesis, which may lead to ASD and other neurological disorders. Furthermore, since not all of the ASD-risk genes are directly connected to each other, this could suggest that there are multiple ways in which ASD can arise from disruptions among these synaptic plasticity genes.

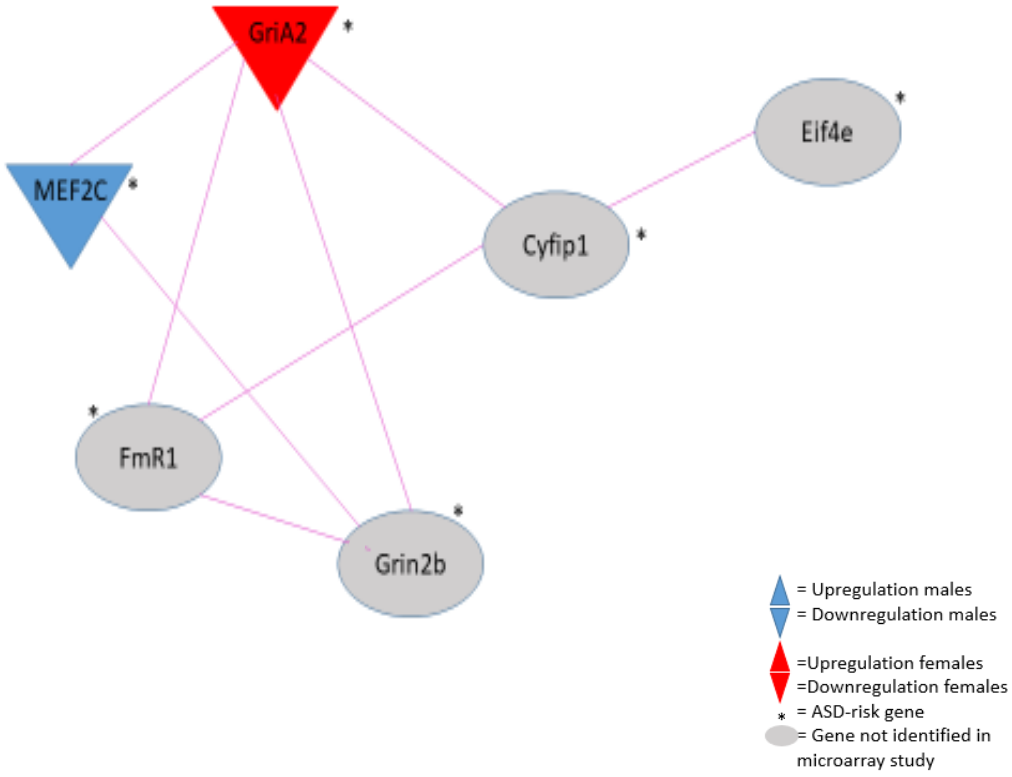


Figure 7. Interaction of gene expression for genes associated with synaptic plasticity based on differential gene expression determined from the microarray analysis of COX-2$^{-/-}$ mice at E16. The genes found in the whole genome microarray study for the COX-2$^{-/-}$ model which were linked to synaptic plasticity at E16, were used to create interaction networks, using GeneMania. Genes dysregulated in females are identified with a red triangle and genes dysregulated in males are identified by blue triangles. Upward facing triangles symbolize an upregulation of the gene expression, whereas downward facing triangles represent a downregulation. Differential gene expression was determined by comparing expression from COX-2$^{-/-}$ mice and wild-type mice. Grey ovals indicate that the gene was not listed as differentially expressed from the microarray analysis. Genes listed were not found to be differentially expressed at E19 in the microarray analysis. ASD-risk genes are identified with an asterisk. Lines connecting genes indicate that both genes play roles in similar pathways.

4.1.5- Analysis of the interaction between genes associated with mitochondrial function, inflammatory responses and synaptic plasticity, generated with Cytoscape, for COX-2-/- knockout mice

There are many genes which have been identified to be associated with mitochondrial function, inflammatory responses, and synaptic plasticity. However, many of these genes could also be connected to genes which are primarily involved in different functions. This would indicate that many of the dysregulated genes identified above could also influence other functions, which are characteristic of ASD and other neurological disorders. This would provide evidence that many of these dysregulated genes could contribute to neurological disorders in multiple ways.

Once the interaction between genes from the mitochondrial function, inflammatory response and synaptic plasticity groups were evaluated separately (above), my goal was to evaluate whether there were genes within each group that interact with genes from other groups. This evaluation was done by inputting the 22 genes outlined above, into Cytoscape's GeneMania to generate an interaction plot containing all genes from each functional. This interaction plot is demonstrated in Figure 8.

The purpose of this analysis was to determine if genes from one group (mitochondrial function, inflammatory response or synaptic plasticity) interacted with genes from another group. This is of interest because it could provide insight into the other functions of these genes, which can have lasting impacts on the brain and lead to neurological disorders, not just based on abnormalities to mitochondrial function, inflammatory responses and synaptic plasticity. This analysis provides insight into the many other pathways in which the 22 genes listed in Figure 8 can play a role in. This will later be outlined in the discussion.

In Figure 8, the genes in the red ovals are associated with mitochondrial function, whereas genes in the yellow ovals are associated with inflammatory response and genes in the purple ovals are involved in synaptic plasticity. Genes that were identified to be interconnected with another gene from a different functional group are connected with a black line.

In Table 2, the number of genes from each group which are connected to genes from another group are depicted. Many genes from each group are connected to genes from both of the other groups, whereas some genes from a given group are not connected to genes from any other group. This is the reason why the genes listed in each column do not add up to the number of genes in total. The purpose of this table is to further convey the data illustrated in Figure 8, by providing a count of the exact number of group-to-group gene interactions.

Interestingly, many genes interact with genes from other groups. The number of interactions between each group is highlighted in Table 2. It can be observed that there are 21 gene connections between the three different groups. For example, of the genes associated with mitochondrial function, *ATP5e, Ndufb10, Sdhc, EiF4ac and TSC2* are connected to genes from the inflammatory response group. Furthermore, *TSC2 and EiF4aC* are connected to genes in the synaptic plasticity group. Therefore, from the mitochondrial function group of genes, 5 genes are connected to genes from the inflammatory response group and 2 genes are connected to genes from the synaptic plasticity group. This means that 5 of the 8 mitochondrial function genes are connected to genes from other groups.

In the case of the inflammatory response genes, *TGFBR1, Acvr1, NFiX, TSC1 and PrKcB* are connected to several genes in the mitochondrial function group, whereas *TGFBR2, Trp53, TGFBR1, and Acvr1* are connected to genes from the synaptic plasticity group of genes. Therefore, 5 genes are connected to mitochondrial function genes and 4 are connected to

synaptic plasticity. As depicted in Figure 8, 5 genes are connected to genes from just one other group and 2 genes are connected to genes from both other groups. Therefore, out of the 8 inflammatory genes, 7 are connected to genes from the other groups.

Finally, for the genes involved in synaptic plasticity, *Fmr1, Grin2b, EiF4e, GRiA2, and MEFC2* are connected to genes from the mitochondrial function group, whereas *Fmr1, GRiA2 and MEFC2* are connected to several genes from the inflammatory response group of genes. This indicates that 5 genes are connected to those in the mitochondrial function group and 3 are connected to the inflammatory response group. More specifically, Figure 8 illustrates that 2 of these genes are connected to genes from one group, whereas 3 are connected to genes from both other groups. Therefore, 5 out of the 6 synaptic plasticity genes are associated with genes from the mitochondrial function and/or synaptic plasticity group.

Based on the data outlined in Figure 8 and Table 2, it has been observed that 17 of the 22 genes involved in this study are connected to several genes from other groups. These genes are, *ATP5e, Ndufb10, Sdhc, EiF4aC, TSC2, TGFBR1, Acvr1, NFiX, TSC1, PrKcB, TGFBR2, Trp53, Fmr1, Grin2b, EiF4e, GRiA2, and MEFC2*. From this data, it can be suggested that many of the genes generated from the microarray analysis and GeneMania, are connected to multiple pathways and they can contribute to down or upstream effects on several mechanisms. For example, some of the interconnections outlined in Figure 8 have been involved in many pathways such as the PKA, mTOR, MAPK, AKT and ERK signalling pathways. Some specific gene interactions which contribute to these pathways will be further elaborated on in the discussion.

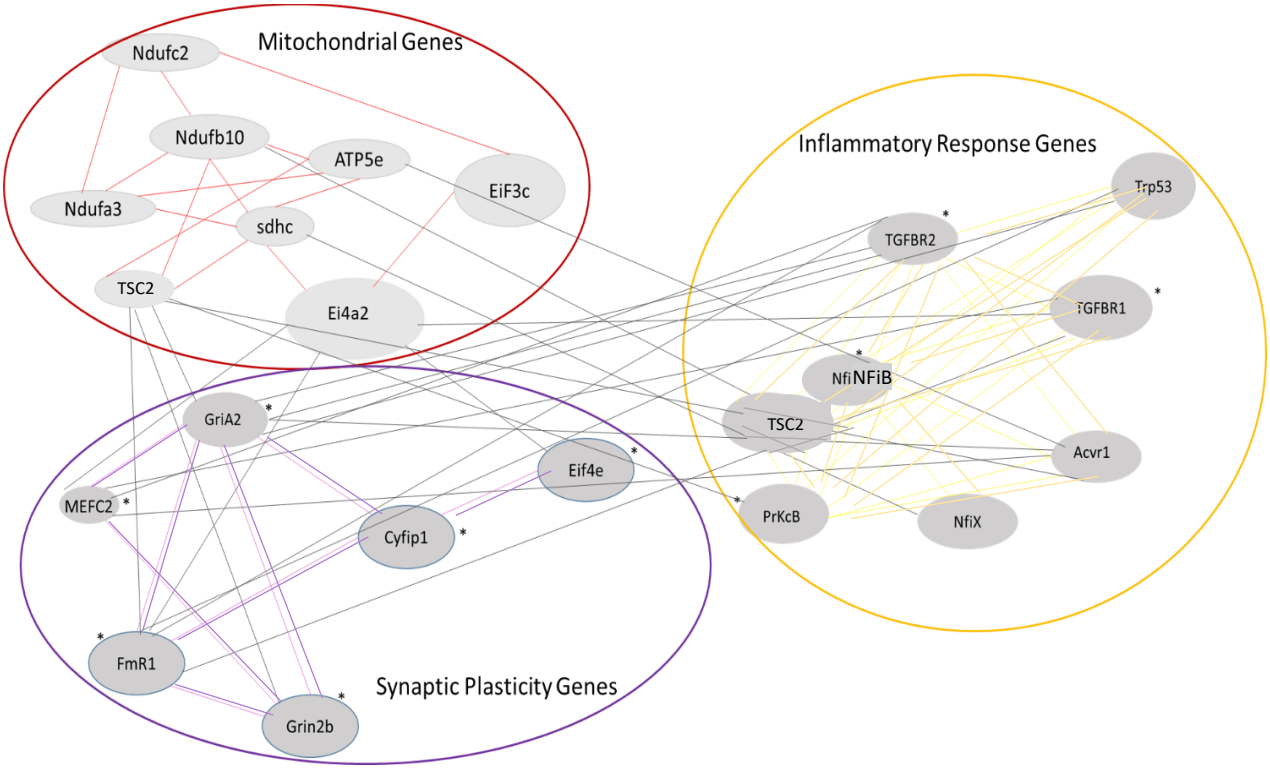


Figure 8. Gene interaction plot generated using GeneMania for all genes found to be associated with mitochondrial function, inflammatory response and synaptic plasticity, based on genes found to be differentially expressed in microarray analysis of COX-2$^{-/-}$ mice at E16 and E19. An interaction plot was generated for all the genes previously found to be associated with mitochondrial function, inflammatory response and synaptic plasticity. Using GeneMania all the interactions between genes from the three functional groups were identified. Genes in a red oval are associated with mitochondrial function, whereas genes in a yellow oval are associated with inflammatory response and genes in a purple oval are involved in synaptic plasticity. Genes interconnected with another gene from a different functional group are connected with a black line. Genes highlighted with asterisks represent ASD-risk genes.

Table 2. Count of the number of genes associated with genes from another functional group based on interaction plots generated with GeneMania. The number of genes which are connected to a gene from a different functional group. The total number of genes affected in each functional group is noted, then the number of these affected genes which are connected to another gene from a different functional group is also noted. The interaction plot from GeneMania was used to perform the count (Figure 8). Some of the genes are connected to no other functional group, whereas some are connected to one other functional group or both.

Functional group	**Total # of affected genes per group**	**# Related to mitochondrial function**	**# of genes connected to Inflammatory Responses**	**# of genes connected to Synaptic Plasticity**
Mitochondrial Function	8 (See Figure 5)	-	5	2
Inflammatory Response	8 (See Figure 6)	5	-	4
Synaptic Plasticity	6 (See Figure 7)	5	3	-

4.1.5- Analysis of gene properties and associated pathologies for genes gathered from the COX-2-/- knockout mice microarray analysis and bioinformatics analysis

Following the completion of the GeneMania analysis, and once the 22 genes (*Ndufa3, Ndufa2, Ndufb10, ATP5e, Sdhc, EiF3c, EiF4aC, TSC2, TGFBR1, Acvr1, NFiB, NFiX, TSC1, PrKcB, TGFBR2, Trp53, Fmr1, Grin2b, EiF4e, GRiA2, cyfip1 and MEF2*) were gathered from the previous analysis shown in Figures 5-7, DAVID Bioinformatics software program was used once again. This program combined with the KEGG plugin was used to determine the official gene name of each gene, the human cytoband, the biological processes associated with the gene and gene ontology, as well as diseases associated with each gene. After this, all the genes which were determined to be connected to ASD were investigated on the Autism Database website and the number of publications connecting each ASD-risk gene to ASD, was noted. All of this information is depicted in Table 3.

From Table 3 it can be seen that of the 22 genes investigated, 17 of them are associated with ASD. However, all the genes investigated play a role in various neurological disorders. Some of these include, Cowden's syndrome, Leigh's syndrome, Noonan syndrome 6, Soto syndrome, Marfan's syndrome and Fraumeni syndrome. These syndromes have been connected to neurological disorders and disruptions in the development of a healthy nervous system.

There were many other neurological disorders which were related to dysregulations in the genes listed in Table 3. These genes were colour coded in Table 3, to illustrate that many genes were observed to play a role in the same neurological disorders, as genes from different functional groups. For instance, many genes were linked to Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), Schizophrenia disorder (SZCD),

Amyotrophic lateral sclerosis (ALS), Fragile-X syndrome (FXS), Autism spectrum disorder (ASD) and learning disabilities (LD). Interestingly, many of the genes associated with ASD are also connected to LD and FXS. This is because these disorders are very closely For instance, children with ASD are also known to have some type of LD. Another example is the connection between FXS and ASD. Research has connected the genetic condition, FXS, with ASD. This is because children who have been diagnosed with FXS are more likely to develop ASD (Devitt et al., 2015).

Furthermore, it is possible that in many of the disorders mentioned above, the abnormal biological processes result from dysregulations of the same genes. For example, the connection between ASD and FXS syndrome is demonstrated in this study because in Figure 8, we seen that there is a connection between several genes associated with FXS and those associated with ASD, in fact some key genes associated with FXS are also known ASD-risk genes. These connections will further be discussed.

Additionally, Table 3 provides some insight into some possible sex-biased gene expressions. The 12 differentially regulated genes found belong to the similar biological processes with 5 dysregulated in males (*Ndufb10, TSC2, NfiB, TGFBR2, and MEFC2)* and 7 in females (*Ndufc2, ATP5e, Sdhc, Eif3c, Eif4a2, and GriA2*). There were no differentially expressed genes in both males and females. This indicates that there is a sex-bias. However, although different genes were dysregulated in each sex they belong to similar biological processes and can potentially result in autism-related pathologies manifested differently in males and females.

Furthermore, there are 10 genes identified in Table 3 to be associated with the 12 genes identified from the microarray. The 10 genes are *TSC1, PrkcB, Nfix, TGFBR1, Acvr1, Trp53,*

Fmr1, Grin2b, CyfiP1, and Eif4e, which are written in black in Table 3. These genes were identified using Cytoscape to be connected to the genes identified to be dysregulated by the microarray analysis, since these 10 genes also play roles in mitochondrial function, inflammatory responses, and synaptic plasticity. Many of these genes have been noted to contribute to biological processes by working alongside the genes identified by the microarray analysis. These specific pathways will be discussed further.

Table 3. Gene information gathered from DAVID Bioinformatics and the Autism Database (AutDB) for all the genes found to be associated with mitochondrial function, inflammatory response and synaptic plasticity, based on differentially expressed genes in the COX-2$^{-/-}$ microarray analysis. DAVID Bioinformatics was used to gather the gene name, human cytoband, biological function, and associated diseases for each of the genes previously determined to be associate with mitochondrial function, inflammatory response and synaptic plasticity. These observations are based on the microarray analysis. Many neurological disorders were observed to be related to the genes such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), Schizophrenia disorder (SZCD), Amyotrophic lateral sclerosis (ALS), Fragile-X syndrome (FXS), Autism spectrum disorder (**ASD**) and learning disabilities (LD). These disorders were colour coded to display similarities between the genes. All ASD-risk genes are underlined and the number of publications found for each ASD-risk gene using the AutDB was noted as well. Genes written in blue were observed to be dysregulated in males, and genes written in red were observed to be dysregulated in females. Genes written in black were identified using Cytoscape to be associated with the genes identified by the microarray analysis and to also play a role in mitochondrial function, inflammatory responses, or synaptic plasticity.

Gene	GeneName	Human cytoband	DAVID Biological Process	Diseases associated with gene	# of publications on AutDB
Ndufa3	NADH: ubiquinone oxidoreductase subunit A3	19q13.42	-Oxidation-reduction (GO:0005739)	AD, PD, HD, SCZD	-
Ndufc2	NADH: ubiquinone oxidoreductase subunit C2	11q14.1	-Oxidation-reduction, -mitochondrial electron transport (GO:0005739)	AD, PD, HD	-
Ndufb10	NADH: ubiquinone oxidoreductase subunit B10	16p13.3	-Oxidation-reduction (GO:0005739)	AD, PD, HD	-

ATP5e	ATP Synthase Epsilon Chain E	20q13.32	-ATP synthesis, -Proton transport (GO:0000275)	AD, PD, HD, Leigh syndrome. **ASD**	**6**
Sdhc	Succinate Dehydrogenase Complex Subunit C	1q23.3	-Oxidative phosphorylation (GO: 0005739)	LD, **ASD**, Cowden's Syndrome	**8**
Eif3c	Eukaryotic Translation Initiation Factor 3 Subunit C	16p11.2	RNA transport (GO: 0005737)	AD, PD, HD,**ASD**	**6**
Ei4fa2	Eukaryotic Translation Initiation Factor 4 Subunit A2	3q27.3	mRNA binding and initiation (GO: 0005737)	AD, PD, **ASD**, Noonan Syndrome 6	**6**
TSC2	Tuberous Sclerosis 2 Protein	16p3.3	-Wnt signalling, -TOR regulation (GO: 0005639)	AD, **ASD**,SCZD,LD	**83**
TSC1	Tuberous Sclerosis 1 Protein	9q34.13	-mTOR signalling(GO: 0005639)	AD, LD, **ASD**, Cerebral Cortical Dysplasia	**36**
PrkcB	Protein Kinase C Beta	16p12.7	-NFkB signalling, -transport, -Calcium signalling (GO: 005737)	AD, **ASD**, LD	**5**
Nfix	Nuclear Factor IX- Type	19p13.13	-DNA transcription, -translation (GO: 0005634)	**ASD**,PD, Soto Syndrome	**17**
NfiB	Nuclear Factor IB- Type	9P23.3	-Neuron axon guidance, -neural cell differentiation (GO: 0005634)	**ASD**,PD, Macrocephaly	**63**
TGFRB2	Transforming Growth Factor B Receptor 2	3p24.1	-MAPKK cascade, -cytokine interaction (GO:0005737)	**ASD**, Marfan's Syndrome	**4**

TGFBR1	Transforming Growth Factor B Receptor 1	9q22.33	-MAPKK cascade, -cytokine interation (GO:0005886)	**ASD**, Marfan's Syndrome	**9**
Acvr1	Activin A Receptor Type 1	2q24.1	-Ligand Binding (GO:0005886)	HD, Brainstem Cancer	-
Trp53	Tumor protein 53	17p13.1	-Tumor surpression, -apoptosis (GO:0005634)	SZCD, Li-Fraumeni Syndrome, Cancer	-
MEF2C	Myocyte Enhancer Factor 2C	5q14.3	-Regulation of synaptic plasticity, -MAPK cascade, -transcription (GO:0005737)	**ASD**, LD,Cancer	**60**
GriA2	Glutamate Receptor Ionotropic AMPA Type 2	4q32.1	-Regulation of long term potentiation (GO:0005886)	SCZD,AD,HD, PD, FXS, **ASD**, ALS	**33**
FmR1	FMRP Translational Regulator 1	Xq27.3	-Regulation of dendritic spine development (GO:0005634)	FXS, LD	**35**
Grin2b	Glutamate Receptor Ionotropic NMDA Type 2B	2p13.1	-Neuroactive ligand interaction, -Nitric oxide signalling	ALS, HD, ADHD,SCZD, **ASD**	**167**
CyfiP1	Cytoplasmic FMR1 Interacting Protein 1	15q11.2	Translation repression (GO:0005773)	FXS, **ASD**, SCZD	**9**
Eif4e	Eukaryotic Translation Initiation Factor 4E	4q23	-Translation regulation (GO:0005737)	**ASD**, FXS	**8**

4.2 Study 2: The effects of PGE_2 on the production of superoxide anion and nitric oxide, in neuronal cells, as an indication of the effects of PGE_2 on ROS production.

4.2.1 Fluorescence intensity measurement resulting from superoxide production in four conditions of differentiated NE4C cells, detected using MitoSox Red.

Superoxide was detected using MitoSox Red (Thermo Fisher Scientific), in differentiated NE-4C cells. In the following experiments, four different conditions were tested to see how fluorescence intensity (FI) changes based on treatment. The four treatments are, the untreated cells (negative control), 1µM PGE_2, 10µM PGE_2 and H_2O_2 (positive control). Before imaging was conducted, cells were incubated with PGE_2 (30 minutes) or H_2O_2 (1 hour), along with MitoSox Red (the last 30 minutes before imaging). Following this, the dye was washed from the cells using PBS, and imaging occurred while the cells were coated with 3mL of PBS. Fluorescence was detected at 510nm, with images being taken every 30 seconds for 2 hours.

As seen in Figure 9, there are suspected difference between the treatments. It is observed that the treatments have similar levels of fluorescence for the first 40 minutes of imaging, but after this point there are significant deviations between the amounts of fluorescence seen between the treatments and the trends each plot follows.

Looking at Figure 9, we can observe several trends in how each treatment altered the FI of MitoSox Red. For instance, it was observed that untreated cells have the lowest amount of average fluorescence. . In this study we used arbitrary units per minute (a.u/min) to quantify fluorescence, as in previous literature with MitoSox Red also applied in cell cultures (Ghanian et al., 2018). The untreated control cells show a gradual increase in superoxide production during the first 60 minutes, then a peak FI of 980 (a.u/min), followed by a decrease to the basal level at 120 minutes of 687 a.u/min. Next, 1µM PGE_2 shows a steady increase in FI, where the intensity

steadily increases to a maximum FI of 1035 a.u/min at 85 minutes and reaches a plateau for the remaining time. The 10µM PGE_2 treatment has an increased amount of fluorescence compared to the untreated cells. For the 10µM PGE_2 treated cells, a gradual increase of FI to a maximum of 1465 a.u/min after 2 hour of imaging was observed. H_2O_2 was used as a positive control for detection of superoxide with the MitoSox Red dye with a FI of 2385 a.u/min after 2 hours of imaging, which appeared to still be increasing after the 2 hours.

If we compare the maximum FI values for each PGE_2 treatments with the untreated control, we observed that the 10µM PGE_2 treatment had a much higher maximum FI (1465 a.u/min after 2 hour) than the 1µM PGE_2 treatment (1035 a.u/min at 85 minutes). This suggest that there are differences in the mechanism which the different concentrations of PGE_2 may cause. For example, these observations suggest that the affinity and activation of EP receptors differs between 1µM and 10 µM PGE_2. Based on these results, it seems that the 10 µM PGE_2 treatment activates the EP receptors longer than the 1µM PGE_2 treatment, since the 10 µM PGE_2 treatment continues to generate superoxide, much longer than the lower dose of PGE_2. This indicates that 1µM PGE_2 likely deactivates the EP receptor activity sooner than a higher concentration. This alludes to a dose-dependent effect between 1µM and 10 µM PGE_2.

The data illustrated in Figure 9 shows that treatment with PGE_2 on differentiated NE-4C cells results in the production of superoxide anion in a dose-dependent manner. In order to determine if these are significant differences between the treatments, statistical analysis will be conducted.

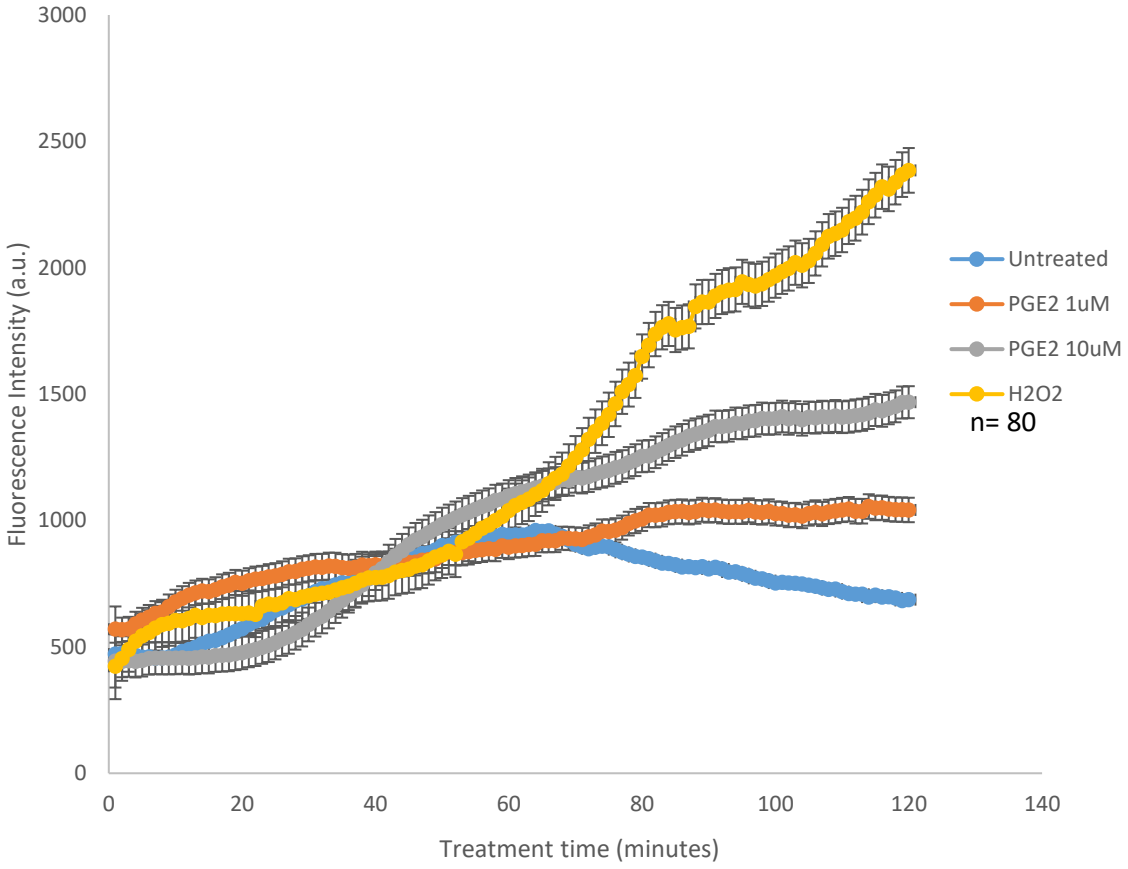


Figure 9. Average production of superoxide anion from the mitochondria of neuronal cells using MitoSox Red dye. Data presented is the average fluorescence (n=80) for four different treatments with SEM bars. Cells were treated with 1 or 10µM of PGE_2 for 30 minutes, H_2O_2 for 1 hour, or left untreated. The fluorescence represents the amount of superoxide generated within the mitochondria of the cells, followed by 30 minutes of incubation with the dye. There is no dye left on the plate when imaging occurs, therefore fluorescence does not begin at 0 arbitrary units (a.u/min).

4.2.2 Analysis of concentration of superoxide produced in differentiated NE-4C cells, detected using MitoSox Red

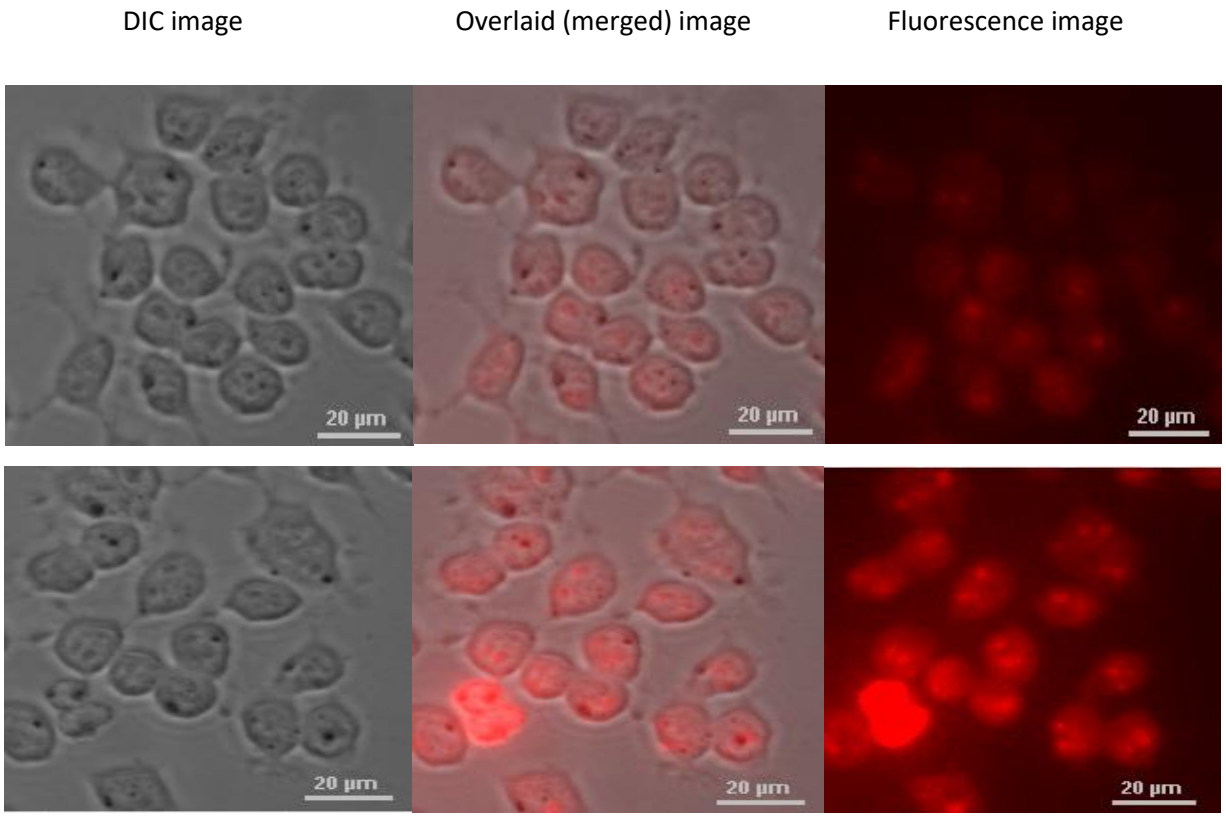
Randomize block analysis was conducted to test whether PGE_2 increases the production of superoxide anion, in a dose-dependent manner. Following the fluorescence microscopy used to detect superoxide production, a randomized block analysis was run to determine if there was a significant difference in the amount of superoxide produced between the four different treatments. This was done by finding the maximum point of fluorescence for each of the 80 cells measured per condition. The 80 values were then averaged, to give the average maximum fluorescence per treatment. This is a different value from the one mentioned above, as the values in Figure 4, were collected from averaging all of the FI values of the 80 cells (80 FI values were averaged per each of the 240 images taken) per treatment, whereas the values used for the randomized block test, only use the maximum FI for each cell imaged (the maximum FI value for each of the 80 cells was averaged, at any time point where that maximum occurred). From here, a randomized block design was used along with a Tukey's post hoc, to determine if there was any significant difference between treatments. This is demonstrated in Figure 10. Representative fluorescent images from untreated cells and 10µM PGE_2 treated cells are shown in Figure 10A

Following imaging, statistical analysis was done to determine if there was a significant difference in fluorescence as a representation of superoxide concentration, between the treatments. Significance was determined based on the average maximum fluorescence (Figure 10B). Based on the randomized block design and Tukey's post hoc, it was determined that the maximum FI from the 1µM PGE_2 and 10µM PGE_2 treated cells was significantly higher than the basal level in the untreated control ($p<0.001$ in both cases). The maximum FI for 1µM PGE_2 was

983 a.u/min, whereas the maximum FI for 10µM PGE_2 was 1446 a.u/min. There was also a significant difference in superoxide concentration between 1µM PGE_2 and 10µM PGE_2 treated cells ($p<0.001$). Also, it was noted that H_2O_2 (control), showed a significant difference compared to the untreated cells ($p<0.001$).

Based on these results, it can be concluded that the two PGE_2 treatments did significantly altered the concentration of superoxide produced. Furthermore, there was a significant difference between the amount of superoxide produced following treatment with 1µM or 10µM of PGE_2 indicating that there is dose-dependent affect in the amount of superoxide produced due to PGE_2.

A.


DIC image
Overlaid (merged) image
Fluorescence image
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm

B.

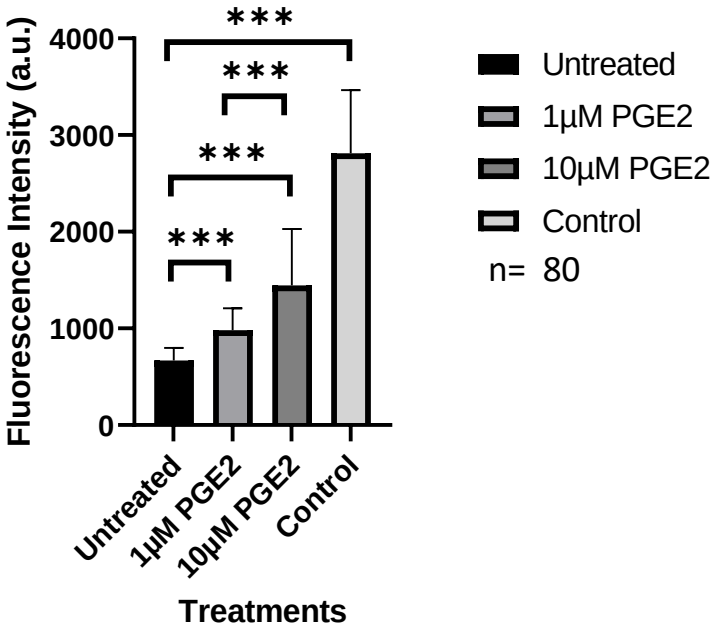

***
***
***
***
4000
3000
2000
1000
0
Fluorescence Intensity (a.u.)
Untreated
1µM PGE2
10µM PGE2
Control
Treatments
Untreated
1µM PGE2
10µM PGE2
Control
n= 80

Figure 10. Superoxide production in response to PGE_2 treatment detected using MitoSox Red from the mitochondria of differentiated NE-4C cells. Fluorescence images taken of untreated cells after 30 minutes incubation with 5μM MitoSox Red dye (top). Images taken of cell treated with 10μM PGE_2 and 5μM MitoSox Red for 30 minutes, which was then washed and removed with PBS before imaging. Both images were taken after one hour of imaging using the TRITC filter. **B.** O_2^- production was measured using time-lapse microscopy. After treating the cells with either H_2O_2 for 1 hour, 1μM or 10μM PGE_2 for 30 minutes, cells were imaged with the MitoSox Red fluorescent dye for 1 hour. Data represented is the mean of the maximum (maximum FI obtained from 80 cells from 3 independent biological replicates, therefore n=80) fluorescence for each condition from three independent experiments with SEM bars. The statistical significance was determined using a randomized block test, followed by Tukey's post hoc comparison. ***$P<0.001$.

4.2.3 Analysis of kinetic rate of superoxide production in differentiated NE-4C cells, detected using MitoSox Red

The kinetic rate of superoxide production was determined as a ratio between fluorescence and time. Following the analysis of the concentration of superoxide production, mentioned in the previous section, the kinetic rate was determined for each treatment. This was done by identifying the point of inflection of each plot generated from the intensity of fluorescence over time. From there, the maximum slope of each plot was found. This maximum slope is the kinetic rate of each plot. The kinetic rate is a quantity which is used to indicate a ratio of how much superoxide is being generated per minute. The rate was found for each of the three replicates per each treatment, which were then averaged, in order to give an average rate for each treatment. This information was used to determine how much superoxide is being generated per minute, which can be useful for future studies.

Following the concentration analysis, kinetic rates were determined by finding the point of infection for each treatment, then the slope (as mentioned above). As seen in Table 4, the 10µM PGE_2 treatment (18.90 a.u/min) was seen to have the highest kinetic rate, followed by the 1µM PGE_2 treatment (13.87 a.u/min), and then the untreated cells (11.71 a.u/min). Since the rate is a ratio between the amount of superoxide produced and time, a higher kinetic rate value indicates that more superoxide is produced in a shorter time.

Based on the values in Table 4, unpaired T-test were conducted to determine if there is a significant difference in the kinetic rate of each treatment. Comparing the untreated cells and the 1µM PGE_2 treatment (11.71 a.u/min and 13.87 a.u/min), there is a significant difference in the kinetic rate of the untreated cells and 1µM PGE_2 treatment ($T= 2.935$, $P<0.004$). Following this, there was a comparison made between the untreated cells and 10µM PGE_2 treatment (11.71

a.u/min and 18.90 a.u/min). It was determined that there is a significant difference in the kinetic rate of the untreated cells and 10 µM PGE_2 treated cells (T= 12.63, P<0.001) Also, when comparing the kinetic rate of both treatments of PGE_2, it was seen that there is a significant difference in the kinetic rate between the 10µM PGE_2 treatment and the 1µM PGE_2 treatment (T=6.082, p<0.001). These findings are illustrated in Figure 11.

Therefore, the results indicate that as the concentration of PGE_2 increased, the rate of superoxide production increased as well. This means that as the concentration of PGE_2 increased, the amount of superoxide which was produced over time, also increased in a shorter time. Therefore, the 10µM PGE_2 treated cells produced more superoxide faster than any other treatment group. This also indicates that there is a dose-dependent effect in the rate by which superoxide is produced, between the 1µM PGE_2 treated cells and 10µM PGE_2 treated cells.

Table 4. Average kinetic rate of differentiated neuronal cells determined using MitoSox Red dye. Kinetic rates were determined by finding the maximum slope from the plots of each treatment. For each treatment, the kinetic rates from each of the 3 replicates was averaged (n=3). The kinetic rates represent the ratio of the intensity of fluorescence over time (a.u/min).

	Untreated cells	1μM PGE_2	10μM PGE_2
Average kinetic rate (a.u/min)	11.71	13.87	18.90

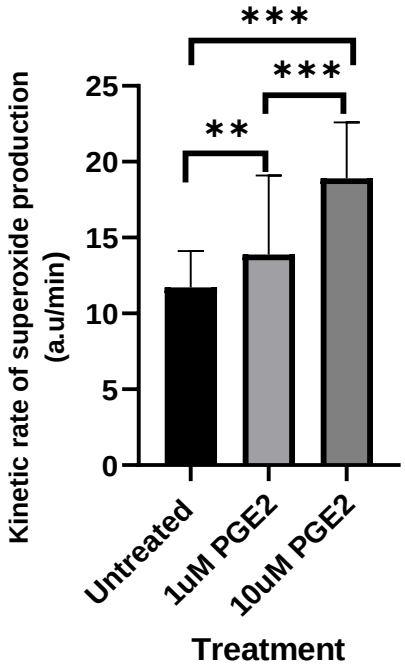


Figure 11. Kinetic rates of superoxide production in response to the treatment with PGE_2 using MitoSox Red. The average kinetic rate determined by finding the maximum slope for each set of data collected from 3 replicates per treatment. Therefore n=3 for each treatment and the statistical significance was determined using an unpaired two-sample T-test. ** $P<0.004$, ***$P<0.001$.

4.2.4 Significance of the results observed from superoxide detection

The results generated from the analysis of the concentration data using MitoSox Red indicate that with the presence of PGE_2, there is increased production of superoxide generated in the mitochondria. This is in line with previous literature in non-neuronal cells suggesting that PGE_2 may play a role in producing ROS (Kosuge et al., 2020). However, our study shows this correlation in neuronal cells for the first time. Furthermore, since there was a significant difference between the intensity of fluorescence between 1μM and 10μM PGE_2, we can state that different doses of PGE_2 result in significantly varying degrees of ROS production, thus indicating a dose-dependent effect.

Based on the data represented in Figure 11 and the significance determined using a two-sample T-test, it was seen that there is a significant difference in the kinetic rates between each treatment. There was a significant difference in the rate of superoxide production between the untreated cells and the 10μM PGE_2 treated cells ($P<0.001$). There is also a significant difference between the untreated cells and 1μM PGE_2 treated cells. This means that both treatments of PGE_2 significantly increased the production of superoxide in a shorter time than the untreated condition. Interestingly, it was also observed that the concentration of PGE_2 influences the rate in a significantly different way, indicating that PGE_2 works in a dose-dependent manner, where a higher concentration of PGE_2 leads to more superoxide being produced faster.

4.2.5 Fluorescence intensity measurement resulting from nitric oxide production in four conditions of differentiated NE4C cells, detected using DAF-2DA

Nitric oxide (NO) production was measured using time-lapse microscopy. DAF-2DA was used as the dye to indicate NO production. Cells were treated with either LPS (positive control) for 2 hours (AdipoGen Life Sciences), 1μM or 10μM PGE_2 for 30 minutes, or they were left untreated (negative control). Following treatment with PGE_2 and incubation, DAF-2DA was added to the cells and imaging commenced. Images were taken every 30 seconds for 45 minutes at 515nm.

As seen in Figure 12, in all conditions there is a gradual increase in the production of NO during the first 15 minutes of imaging. However, each treatment starts at a different fluorescence intensity, since cells were not incubated with dye prior to imaging. Following the first 15 minutes of imaging, the FI begins to plateau for each treatment. The untreated cells show a maximum FI of 800 a.u/min, whereas the 1μM PGE_2 treated cells have a maximum FI of 855a.u/min. 10μM PGE_2 treated cells demonstrate the highest amount of intensity, with a maximum FI of 1345 a.u./min, which is higher than the positive control (LPS) that has a maximum FI of 1130 a.u./min.

These observations from Figure 12 suggest that there may be significant differences in NO production among many of the treatments. There seems to be great difference in the maximum FI values from the plots, therefore statistical analysis must be conducted in order to determine if these differences are significant.

4.2.6 Analysis of concentration of nitric oxide produced in differentiated NE-4C cells, detected using DAF-2DA

Following the fluorescence microscopy used to detect nitric oxide production, analysis was done to determine if there was a significant difference in the amount of nitric oxide produced between the four different treatments. Analysis was conducted by first finding the maximum point of fluorescence for each of the 45 cells measured per condition. The 45 values were then averaged, to give the average maximum fluorescence per treatment. From there, a randomized block design was used along with a Tukey's post hoc, to determine if there was any significant difference from treatment to treatment. This is demonstrated in Figure 13.

From Figure 13, it is illustrated that the maximum FI for untreated cells is 689 a.u/min, which is not significantly different from 1µM PGE_2 treated cells, which had a maximum FI of 791 a.u/min ($p<0.001$). The maximum FI for the 10µM PGE_2 treated cells is 1258 a.u/min, which is significantly different from the untreated cells ($p<0.001$) and the 1µM PGE_2 treatment ($p<0.001$). Also, it was noted that LPS (positive control) shows a significant difference compared to the untreated cells ($p<0.001$).

It can be seen from Figure 13 that 10µM PGE_2 increased the amount of NO produced, as the average maximum fluorescence increased over time. Also, there is a significant difference between untreated cells and cells incubated with LPS, making LPS a suitable positive control. Furthermore, there was not a significant difference between the untreated cells and those treated with 1µM PGE_2. Additionally, there is a significant difference in the amount of NO produced between 1µM PGE_2 and 10µM PGE_2. This indicates that there is likely a dose-dependent effect on NO production induced by PGE_2.

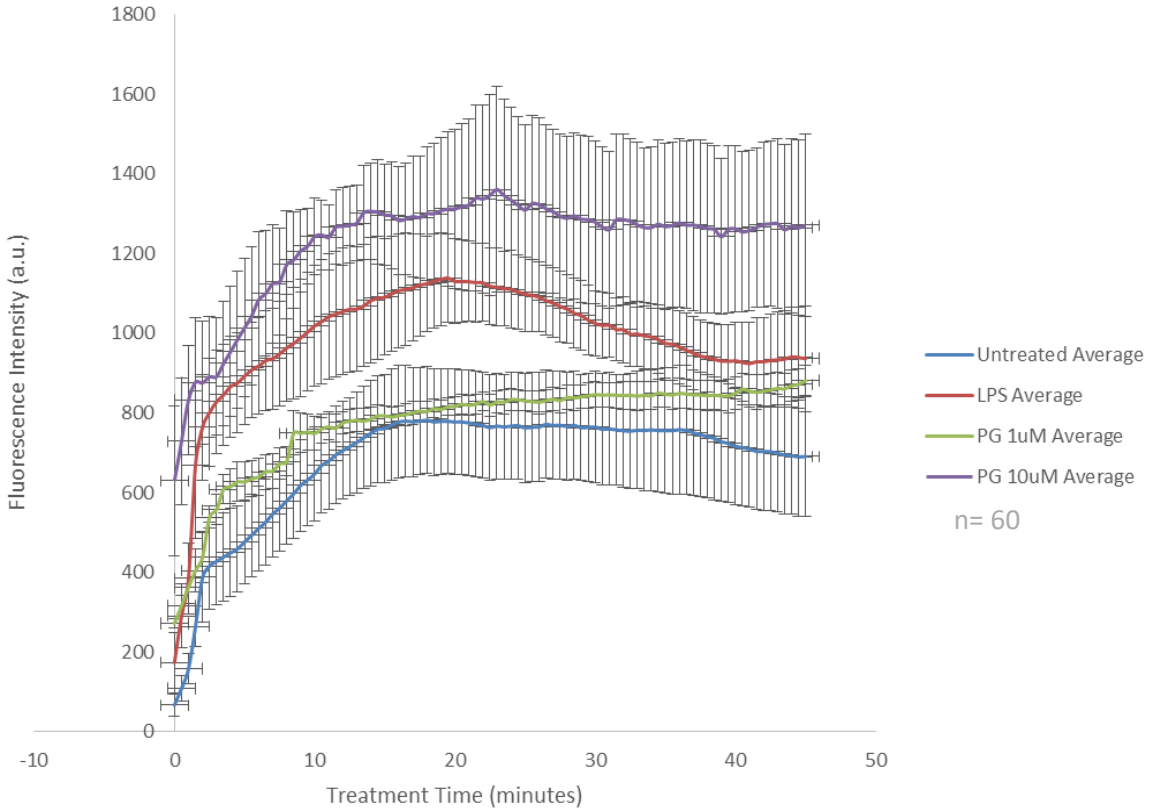


Figure 12. Average production of nitric oxide within the cytosol of differentiated neuronal cells detected using DAF2-DA. Data presented is the average fluorescence (n=45) for four different treatments. Cells were treated with 1 or 10µM of PGE_2 for 30 minutes, LPS for 2 hours, or left untreated. The fluorescence represents the amount of NO generated within the cell, while DAF-2DA is on the plate. Images were taken every 30 seconds for 45 minutes.

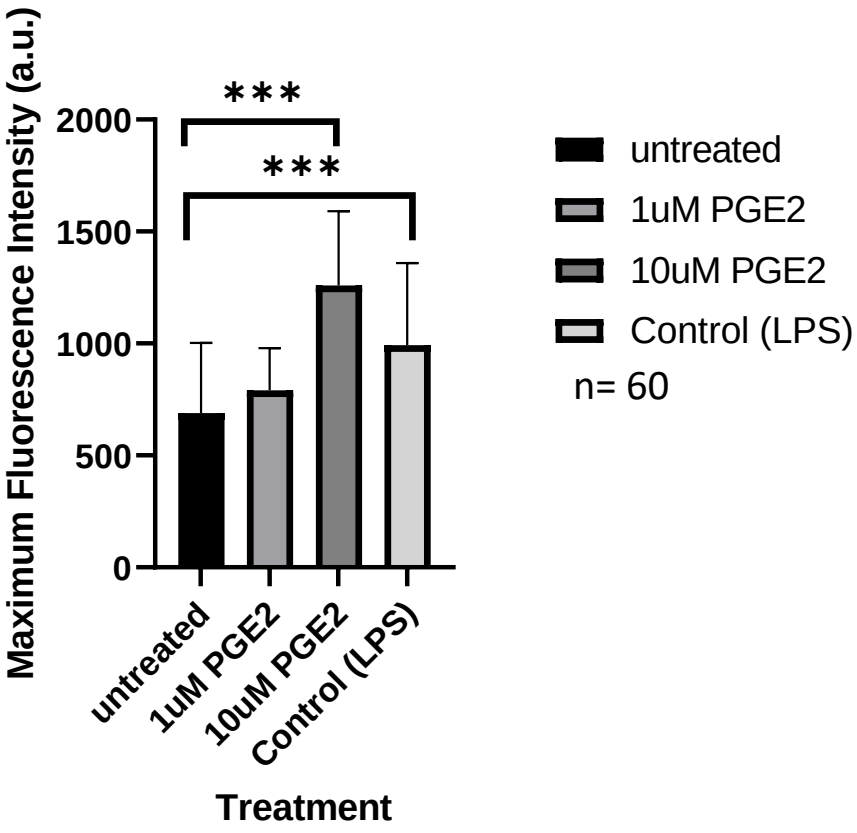


Figure 13. Analysis of nitric oxide production detected within the cytosol of differentiated neuronal cells using DAF-2DA. The data represents the average maximum fluorescence of 45 cells per each treatment, collected using DAF-2DA. A randomized block analysis followed by a Tukey's post hoc test was run, to determine if there was a significant difference, in the amount of fluorescence, between the different treatments. There was shown to be a significant difference between the untreated cells and 10µM PGE_2, and LPS (control). ($p<0.001$)

4.1.7 Analysis of Kinetic rate of nitric oxide production in differentiated NE-4C cells, detected using DAF-2DA

Following the analysis of the concentration of nitric oxide production, the kinetic rate was determined for each treatment. This was done by identifying the point of inflection of each plot and from there, the maximum slope of each plot was found. This maximum slope is the kinetic rate of each plot. The rate was found for each of the three replicates per each treatment, which were then averaged, in order to give an average rate for each treatment. This information will be used to determine how much nitric oxide is being generated per minute and how each treatment can alter this rate.

As seen in Table 5, the 10µM PGE_2 treatment was seen to have the highest kinetic rate (19.57 a.u/min), followed by the untreated cells (14.25 a.u/min), then the 1µM PGE_2 treatment (12.65 a.u/min) had the lowest kinetic rate. This would suggest that 10µM PGE_2 produces more nitric oxide faster than any of the other conditions. Interestingly, this would also suggest that 1µM PGE_2 generates nitric oxide slower than the untreated cells.

Once the kinetic rates were determined, an unpaired T-test was conducted in order to determine if there is a significant difference in the rate at which nitric oxide is produced between the treatments. These results are illustrated in Figure 14. Comparing the untreated cells and the 1µM PGE_2 treatment (14.24 a.u/min and 12.65 a.u/min, respectively), there is no significant difference in the kinetic rate of the treatments. Following this, there was a comparison made between the untreated cells and 10µM PGE_2 treatment (14.24 a.u/min and 19.57 a.u/min, respectively). It was determined that there is a significant difference in the kinetic rate of the untreated cells and the 10µM PGE_2 treatment ($T= 4.132$, $P<0.001$), Finally, when comparing the 1µM PGE_2 treatment and the 10µM PGE_2 treatment, it was determined that there was a

significant difference in the rate of nitric oxide produced ($T=6.091$, $P<0.001$), there was no significant difference in the amount of nitric oxide produced between the untreated cells and 1µM PGE_2 treated cells.

These results indicate that as the concentration of PGE_2 increased, the amount of nitric oxide which was produced over time, also increased, meaning that 10µM PGE_2 treated cells, produced more nitric oxide faster than any other treatment group. Based on these results, it is observed that untreated cells and 1µM PGE_2 treated cells, generate nitric oxide at the same rate. This also means that 10µM PGE_2 treated cells produce nitric oxide at a significantly different rate than 1µM PGE_2 treated cells, resulting in a dose-dependent effect in the rate of nitric oxide production.

Table 5. Average kinetic rate of differentiated neuronal cells determined using DAF-2 DA dye. Kinetic rates were determined by finding the maximum slope from the plots of each treatment. For each treatment, the kinetic rates from each of the 3 replicates was averaged. The kinetic rates represent the ratio of the intensity of fluorescence over time (a.u/min).

	Untreated cells	1μM PGE_2	10μM PGE_2
Average kinetic rate (a.u/min)	14.25	12.65	19.57

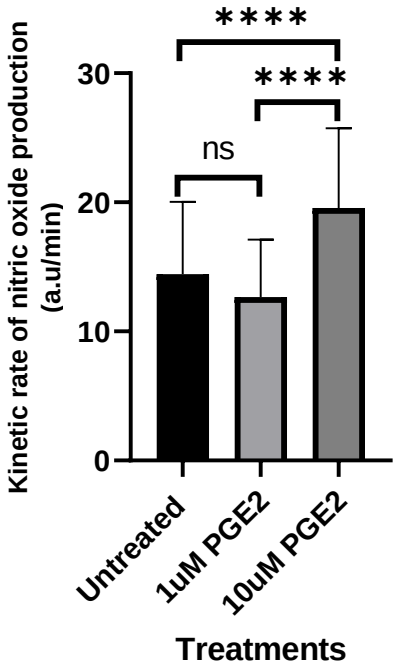


Figure 14. Kinetic rates of nitric oxide production using DAF-2DA. The average Kinetic rate determined by finding the maximum slope for each set of data collected from 3 replicates per treatment. Therefore n=3 for each treatment and the statistical significance was determined using an unpaired two-sample T-test. ****P<0.001.

4.2.8 Significance of the results observed from nitric oxide detection

The results from the concentration analysis indicate that with the presence of PGE_2, there is increased production of nitric oxide generated in the cells. This is significant because with this information we can conclude that PGE_2 plays a role in producing RNS. Also, since 10µM PGE_2, showed an increase in NO production, but 1µM PGE_2 did not show any difference compared to the untreated cells, we can assume that a higher dose of PGE_2 is needed for there to be an effect on the cells. This higher dose of PGE_2 resulted in significantly increased levels of NO since there was a significant difference between the intensity of fluorescence between 1µM and 10µM of PGE_2, thus indicating a dose-dependent effect in production.

This is further illustrated by the kinetic rate analysis, and the significance determined using the unpaired two-sample T-test. It was seen that there is a significant difference in the kinetic rates between each treatment. There was a significant difference in the rate of nitic oxide production between the untreated cells and the 10µM PGE_2 treated cells ($P<0.001$). However, there is no significant difference in the rate of nitric oxide production between the 1µM PGE_2 treated cells and the untreated cells. Another interesting observation is that there is a significant difference in the rate of nitric oxide production between 10µM PGE_2 treated cells and the 1µM PGE_2 treated cells. This indicates that PGE_2 works in a dose-dependent manner, where a higher concentration of PGE_2 leads to more nitric oxide being produced faster. This also demonstrates that there is a threshold concentration which needs to be reached before there is a significant increase in the rate of nitric oxide production, since there was no difference seen at the lower concentration of PGE_2 and the untreated cells.

Chapter 5- Discussion

5.1-Study 1: Identification of genes associated with mitochondrial function, inflammatory responses and synaptic plasticity, using whole genome microarray data from males and females COX2-/- offspring

5.1.1 Overview of Results

In study 1, several key results were noted which provide insight into how altered COX2-PGE_2 signalling can influence gene expression. The COX-2 knock-out mouse model was used to illustrate that deficiencies in COX-2 and PGE_2 can lead to differentially expressed genes, which are important members of pathways associated with mitochondrial function, inflammatory responses and synaptic plasticity. There were several differences seen in gene expression among male and female mice, as well as difference in gene expression at E16 and E19. There were also many ASD-risk genes associated to be a part of the Cytoscape analysis, which are involved in mitochondrial function, inflammatory responses, and synaptic plasticity. Furthermore, many of the genes which were identified using Cytoscape to be primarily associated with either mitochondrial function, inflammatory responses, or synaptic plasticity, were also associated with many brain pathologies. For example, several genes were associated with ASD, AZD, PD, HD, FXS, ALS, and many other syndromes associated with the nervous system.

The results from study 1 provide insight into a possible mechanism in which a COX-2 deficiency can have lasting genetic consequences on the developing brain. Many of the gene dysregulations were observed at only one specific prenatal stage. This is an indication of a possible compensatory mechanism which the developing mouse may undergo between the two stages. For example, there were many genes which were differentially expressed at E16 but not at E19, and vice versa. This can indicate that there was an attempt to compensate for the dysregulation, which led to a shift in gene expression back to WT levels. However, since there

were ASD-risk genes (*Ndufa3, Sdhc, EiF3c, NFiB, TGFBR2, MEFC2, and GRiA2)* which were only differentially expressed at E16 and not at E19, the initial differential gene expression at E16 could possibly have lasting implication on the fetus, even after birth. For example, dysregulation in these ASD-risk genes have been noted to play roles in mitochondrial function, inflammatory responses and synaptic plasticity, all of which have been observed as characteristic features of children with ASD (Goh et al., 2014, Alley et al., 2013, Gilbert et al., 2014).

5.1.2 Interconnected genes play a role in many key developmental pathways

One interesting finding from study 1 was that many genes identified to be associated with mitochondrial function, inflammatory responses, or synaptic plasticity, were also seen to be involved with genes from a different functional group, in other specific pathways. Many of these pathways have implications in several neurological disorders such as, ASD, AZD, PD, ALS, and many others. Therefore, since many of the genes listed in Table 3 are known to be linked to the same disorders as several other genes in the table, it is possible that multiple genes identified in Table 3 may be components of the same pathways which are associated with specific neurological disorders.

For example *Transforming growth factor Beta 1 and transforming growth factor Beta 2* (*TGFBR1 and TGFBR2)* are both associated with *Glutamate ionotropic receptor AMPA type subunit 2* (*GRiA2)*. These genes are ASD-risk genes and together have been noted to all play a role in the *protein kinase A* (PKA) pathway and the *transforming growth factor beta* (TGFB) signalling pathway (Saarienen et al., 2017, Xu et al., 2009). Both pathways play a role in development which may be altered in a fetus due to dysregulations in the expression of genes

such as *TGFBR1, TGFBR2, and GRiA2*. PKA is responsible for crucial signal transduction and has been noted to play a role in learning and memory (Loomis et al., 1998). The TGFB signalling pathway is involved in the timing of many key developmental milestones. For instance, it is responsible for factors associated with cell growth, cell proliferation, cell differentiation and apoptosis (Saha et al., 2017). Both of these pathways are essential for neurodevelopment as they are responsible for initiating many key milestones involved in healthy brain and nervous system development (Meyers et al., 2017, Jason et al., 2010, Saha et al., 2017). Due to the many biological functions that the PKA and TGFB signalling pathways are a part of, *TGFBR1, TGFBR2, and GRiA2* also play roles in many other downstream cascades. For example, evidence has suggested that mutations in these 3 genes can lead to apoptosis, increased cell proliferation and inhibition of tumour supressing factors (Hu et al., 2014, Zhang et al., 2017, Pache et al., 2014). These factors have widely been associated with cancer, and these three genes have been noted to be mutated in many cancers such as, breast cancer, lung cancer, colorectal cancer, endometrial cancers, among others (Hu et al., 2014, Zhang et al., 2017, Pache et al., 2014).

Another pathway which is activated upstream by the gene expression of *TGFBR2 and Myocyte enhancer factor 2c (MEFC2)* is the P38 subclass of the *mitogen-A protein kinase* (MAPK) pathway (Sapkota et al., 2013). MAPK signalling has been observed to play a crucial role in neurodevelopment, by promoting differentiation of stem cells into their final fate in the central nervous system (Albert-Gresco et al., 2012). MAPK has also been noted to play a role in long-term potentiation (LTP) and learning and memory (Houscott, 2008). *TGFBR2* was noted to be involved in inflammatory responses in study 1 whereas *MEFC2* was seen to be involved in synaptic plasticity. However, these genes can interact with one another to influence the activation of the P38 MAPK signalling pathway (Schroen et al., 2016). *TGFBR2* gene expression

plays a role in phosphorylating and activating the enzyme TAK1 (also known as mitogen-activated protein kinase 7, MAP3K7), which then can activate the transcription factor *MEFC2*, which thus can alter the p38 MAPK signalling (Sapkota et al., 2013). The p38 subclass of the MAPK signalling pathway has been associated with certain brain pathologies, such as Parkinson's disease and ASD (Robinson et al, 2018, He et al., 2008).

Furthermore, *Eukaryotic translation initiation factor 4e (Eif4e), Eukaryotic translation initiation factor 4a2 (Eif4a2), Tuberous sclerosis complex subunit 1(TSC1) and Tuberous sclerosis complex subunit 2 (TSC2),* all play a crucial role in the *mammalian target of rapamycin* (mTOR) signalling pathway (Shownat et al., 2014). These genes belong to the three functional groups identified in study 1, but all interact with one another to lead to the activation of the mTOR signalling cascade. mTOR signalling plays a role in neurodevelopment and is also associated with many neurological disorders such as ASD, ADHD, Epilepsy and AZD (Lipton and Sahin, 2015). mTOR is responsible for regulating many neuronal cell functions during development, such as proliferation, migration, and differentiation (LiCausi and Hartman, 2018). Additionally, mTOR signalling contributes to dendritic spine formation and synaptic plasticity (Switon et al., 2017).

Additionally, *Activin receptor type-1 (Acvr1), MEFC2, GRiA2, TSC1 and TSC2* play a role in many subclasses of MAPK signalling (Huang et al., 2008, Poddar et al., 2017, Chen et al., 2017). *Acvr1 and TSC1* are involved in inflammatory responses, *MEFC2 and GRiA2* are involved in synaptic plasticity, whereas *TSC2* is involved in mitochondrial function, as seen in study 1. Together these 5 genes are involved in the AKT, *extracellular-signalling regulated kinase* (ERK) and p38 subclasses of MAPK signalling (Huang et al., 2008). As mentioned

previously MAPK signalling plays crucial roles in neurodevelopment and has been associated with many neurological disorders (He et al., 2008).

Interestingly, there has been a lot of evidence indicating a cross-interaction between mTOR and MAPK signalling (Mi et al., 2009, Rosina et al., 2019). Literature has suggested that as there are abnormalities in mTOR signalling, there are also disruptions observed in the MAPK signalling cascade (Mi et al., 2009). As seen in study 1, there are many genes which are involved in these two signalling pathways, which are connected to each other. Many of these genes are found within the same functional group, such as *Acvr1* and *TSC1* both being a part of the inflammatory response category described in study 1, and *TSC2 and Eif4e* being a part of the mitochondrial function group, and *MEFC2 and Eif4e* belonging to the synaptic plasticity group. However, these genes are also connected to those in other groups as well, which can lead to upstream and downstream effects on mTOR and MAPK signalling (Rosina et al., 2019). Surprisingly, a majority of these genes are also ASD-risk genes, and simultaneous abnormal mTOR and MAPK signalling has been observed to be associated with ASD as well (Rosina et al., 2019, Ganesan et al, 2019).

Therefore, in study 1 there were many genes which were connected to one another from different functional groups that can influence many critical pathways which are associated with neurodevelopment and have also be implicated with several neurological disorders, also identified in study 1.

5.1.3 Mitochondrial Function and COX2-PGE_2 signalling

The findings from study 1 indicate that knocking-out COX-2 and therefore having a deficiency of PGE_2 in mice, leads to many gene dysregulations in the developing fetus. Furthermore, it was observed that several of the genes which were differentially expressed are involved in aspects of mitochondrial function. The dysregulation of many of these genes can have detrimental impacts on the mitochondria. For example, the genes identified in Figure 4 to be associated with the electron transport chain, play key roles in regulating ATP generation and if there are disruptions in this process, there can be increased mitochondrial dysfunction, eventually leading to apoptosis (Chakrabati et al., 2011).

There has been literature discussing that increased levels on PGE_2 results in mitochondrial dysfunction, in many cell types (Zhang et al., 2018, Cillero-Pastor, et al., 2008). However, our results from study 1, also demonstrate that reduced levels of PGE_2 also can result in mitochondrial dysfunction. Literature indicates that the COX2-PGE_2 pathway plays a critical role in mitochondrial activity and appropriate mitochondrial function (Zhang et al., 2018). Increases in the level of PGE_2 have been observed to have many consequences on the mitochondria, including increased leaks to the electron transport chain, decreased mitochondrial membrane potential, and even apoptosis (Chakrabati et al., 2011). The data in study 1 also suggest that decreased levels of PGE_2 is likely to lead to mitochondrial dysfunction, due to

differential gene expression of several genes important for healthy mitochondrial function. Genes such as *Ndufdc, Ndufa3. Ndufb10, and ATP5e*, all play very important roles in the electron transport chain, and ATP generation (Maldonando et al., 2010). Possible dysregulations of these genes can lead to the inability for the cell to generate ATP and even leaks in the electron transport chain, which can lead to increased levels of ROS production in the cell (Maldonando et al., 2010). Therefore, this is one of the first studies to indicate that reduced PGE_2 levels in the brain can lead to mitochondrial dysfunction as well.

Furthermore, the dysregulation of several genes involved with mitochondrial function can have implications on many major signalling pathways. For instance, COX-2 has been observed to alter *TFAM* expression in the mitochondria, which interacts with many other mitochondrial genes, to disrupt the signalling cascade involved in the p38 subclass of MAPK signalling (Burlacu et al., 2003). As mentioned above, several genes identified in study 1, such as *TSC2*, are involved in the MAPK signalling pathway (He et al., 2008). It has also been noted that abnormal levels of PGE_2 can lead to altered AKT phosphorylation, as a result of differential gene expression in the mitochondria. We noted several genes from study 1, which play a role in AKT and MAPK signalling. For example, *TSC2, TSC1 and Acvr1* play direct roles in AKT signalling (Huang et al., 2008). Therefore, the differential gene expression discussed in study 1 can influence the propagation of signalling pathways, such as p38 and AKT subclasses of the MAPK pathway. Further research can be done to indicate exactly which genes from the mitochondrial

function group identified in study 1, are involved in disruptions in MAPK signalling, and how these genes are directly involved in these disruption.

5.1.4 Mitochondrial Function and Neurological Disorders such as ASD

Mitochondrial dysfunction has been associated with many neurological disorders over the years. There are many factors which can contribute to mitochondrial dysfunction. For example, genetic factors, such as the dysregulated gene expression observed in study 1, as well as environmental factors such as, diet, drug use and stress, can all contribute to increased levels of mitochondrial dysfunction (Rose et al., 2014). Mitochondrial dysfunction plays a role in the brain and has been associate with several neurological disorders. For instance, many symptoms associated with increased levels of mitochondrial dysfunction such as, intellectual disability, language and speech impairments, and neuropsychotic behaviours, have also been associated with brain pathologies such as, ASD, BPD, SCD, PD, and AD (Guilivie et al., 2010, Goh et al., 2014). Interestingly, as seen in Table 3, the genes we identified to be associated with mitochondrial function, were also associated with similar brain pathologies, particularly AD, PD, SCD, and ASD.

Literature has tied mitochondrial dysfunction and ASD together for many years now. In many studies there have been observations of many biochemical markers for mitochondrial

function found in post-mortem brain samples of adults and children with ASD (Tang et al., 2013). Furthermore, in one study, 80% of the participating children had indications of mitochondrial dysfunction found through biomarkers in the blood (Chauan et al., 2014). There have been many observations where individuals with ASD exhibit signs of mitochondrial dysfunction, such as mitochondrial DNA mutations and abnormal turnover, disruptions to complex I and III of the ETC, as well as membrane depolarization (Guilivie et al., 2010, Tang et al., 2013, Goh et al., 2014). These findings lead to the idea that ASD and mitochondrial dysfunction are associated with one another (Goh et al., 2014). The data from study 1 suggests that one possible mechanism by which ASD and mitochondrial dysfunction are related, is via gene dysregulation. Many of the genes from study 1, especially those associated with mitochondrial function, were also identified as ASD-risk genes (*Sdhc, EiF3c and ATP5e and TSC2).* These genes were also seen to be differentially expressed at specific stages and among a specific sex. *Sdhc, EiF3c and ATP5e* were observed to be upregulated at E16 in females only, but not differentially expressed at E19. However, *TSC2* was downregulated at E16 and upregulated at E19, in males only. These results could indicate that the difference between these 4 ASD-risk genes, which also happen to be associated with mitochondrial function, could have lasting implications on mitochondrial function in the brain, and may also related to the connection between ASD and mitochondrial dysfunction. Further research would have to be

done in order to determine some of the lasting consequences of differential expression of these 4 genes, in order to determine how closely related they are to ASD and mitochondrial dysfunction.

5.1.5 COX2-PGE_2 signalling and inflammatory response

It has been noted that PGE_2 is a major inflammatory mediator and plays a role in regulating many inflammatory responses. PGE_2 has been observed to play crucial roles in hyperalgesia and pain sensation (Mariyama et al., 2005). In fact it has been noted that PGE_2 is one of the main pro-inflammatory prostanoids and it contributes greatly to nociception (Minami et al., 2001). This suggest that abnormalities in PGE_2 can result in abnormalities in pain perception. PGE_2 is also involved in many pro-inflammatory and anti-inflammatory responses, which are relevant to gene expression (Minami et al., 2001). It has been observed that differentially expressed genes, such as those identified in study 1, can have pronounced effects on inflammatory responses, such as prolonged activation of microglia and cytokines (Minami et al., 2001). Considering the data outlined in study 1, several genes were observed to be associated with inflammatory responses (*NFiB,TGFRB2, TSC1, PrkcB, NFiX, ACVr1, TGFBR1 and Trp53*) and 2 of which *(NFiB and TGFBR2),* were directly downregulated in males at E16, due to PGE_2 deficiencies. This can further contribute to the idea that PGE_2-induced gene dysregulations are closely connected to impairments in inflammatory responses.

Furthermore, it has been observed that PGE_2 plays a role in pain sensation via the PKA and PKC pathways (Sachs et al., 2009, Meevs et al., 2006). As discussed above, several genes from study 1 were associated with this pathway. *TGFBR1 and TGFBR2* were seen to be directly involved in this pathway, and both of these genes were noted to be a part of inflammatory

responses. In fact, *TGFBR2* was also gathered from the microarray data directly, indicating that there is a direct effect from abnormal PGE_2 signalling to a downregulation in *TGFBR2* in males at E16. This data further suggests that abnormal levels of PGE_2 signalling can have a direct impact on the PKA pathway, responsible for pain sensation, possibly via a dysregulation in the *TGFBR* class of genes. It is also noteworthy to mention that *TGFBR2* is an ASD-risk gene. This might suggest a possible connection between pain perception and PGE_2 in individuals with ASD.

5.1.6 Inflammatory Response and ASD

Literature has shown many connections between ASD and abnormal inflammatory responses. Many are even labelling certain aspects of abnormal immune response, such as increased and prolonged cytokine and microglial activation, as a trans-etiology of ASD (Elias et al., 2015). There has been evidence indicating that increased levels of cytokine activity is associated with ASD. For instance, in post-mortem brain tissue samples of children with ASD, there was a widespread of inflammatory markers, such as increased levels of cytokines, throughout different brain regions (Masi et al., 2007). Additionally, there has also been elevated levels of cytokines noted from spinal fluid and blood collected from individuals with ASD. ASD is also closely tied to prolonged microglia activation. Over time this prolonged activation of the microglia and increasing numbers, leads to cell damage which can have serious consequences (Elias et al., 2015, Masi et al., 2007). Prolonged microglial activation has been associated with neuronal cell death, and loss of synaptic connections. In fact, children with ASD have been noted to have severe under connectivity in terms of synaptic plasticity and a large part of this is suggested to be due to microglia activation (Rodrigues et al., 2011). This is interesting because in study 1, we note that many genes associated with inflammatory responses are also associated with synaptic plasticity. Furthermore, many of these genes, such as *TGFBR1 and TGFBR2* are

also associated with ASD. This could suggest that these genes are involved in altering synaptic connectivity and are influenced by microglial activity.

ASD is known to be associated with increased neuroinflammation, which has also been highly associated with genes (Rodrigues et al., 2011). This indicates that the results observed in study 1, may offer some new insight into how PGE_2 is related to neuroinflammation and ASD through specific gene dysregulations. *TGFBR2, NFiB, TSC1, PrKcB, and TGFBR1* were all noted in study 1 to be ASD-risk genes which play important roles in inflammatory responses. It has already been suggested that *TGFBR1 and TGFBR2* are involved in the PKA pathway and synaptic plasticity which suggest that these genes could be associated with pain perception and abnormal synaptic connectivity in ASD. Furthermore, the microarray data used in study 1, indicated that *TGFBR2 and NFiB* are both downregulated in males at E16. This could indicate that this downregulation has lasting implications in ASD and could also indicate a connection between dysregulation in these two genes and the sex-bias we see in the population of individuals with ASD.

As mentioned above, PGE_2 plays a critical role in pain sensation. There has also been recent literature indicating that pain perception may be abnormal in individuals with ASD. Studies have indicated that many children with ASD demonstrate characteristics associated with a higher pain threshold and decreased pain sensitivity than the control group (Alley et al., 2013). However, some children with ASD demonstrate the opposite behaviour, where they seem to have a lower pain threshold and higher pain sensitivity (Alley et al., 2013). This indicates that there is some disruption in the pain sensation mechanism among children with ASD, but it can lead to a variety of implications on how a child with ASD might perceive pain compared to a neurotypical child (Alley et al., 2013). In individuals with ASD it has been noted that there is an impairment

in the perception of pain, due to aspects of the cognitive pathways of pain perception being disrupted in children with ASD (Yasuda et al., 2016). The PKA pathway is noted to be a cognitive pathway, which can be disturbed by abnormal PGE_2 signalling. This could suggest that PGE_2 plays a key role in abnormal pain perception in individuals with ASD, since PGE_2 is heavily associated with both these components.

5.1.7 COX2-PGE_2 signalling and Synaptic Plasticity

Over recent years there has been growing research which investigates the possible connection between COX-2 and synaptic plasticity. Many have noted that COX-2 does indeed alter the degree to which synaptic signalling and synaptic plasticity can be reached in the brain, indicating that COX-2 plays a very important role in neuronal plasticity (Chen et al., 2002). Varying the level of COX-2 signalling which occurs in the brain, has led to evidence that there will be an altered degree of synaptic plasticity and ultimately an interference in the degree of learning and memory which can be accomplished by an individual (Yang and Chen, 2008). It has been noted that if COX-2 is inhibited, there is a significant decrease in synaptic connectivity and learning and memory (Chen et al., 2002). This may be the case since, if there is an elevation or suppression of COX-2 signalling in the brain, there is either enhanced neurotransmission and long-term potentiation, or a decrease in neurotransmission which ultimately leads to a decrease in long-term potentiation, respectively (Yang and Chen, 2008).

The results gathered from study 1 indicate that there were 2 genes differentially expressed due to a COX-2 knock-out in our mice. Interestingly there was a downregulation of *MEFC2 and GRiA2* in males at E16. This supports the idea that as there is a suppression in COX-2 signalling, there tends to be a reduction in synaptic signalling and synaptic plasticity. This is because both of these genes are highly associated with synaptic plasticity, and it has been

shown in previous literature that if there is a decrease in *MEFC2 and GRiA2* expression, then there is also a significant decrease in synaptic plasticity (Barbosa et al., 2008, Yang et al., 2019). *MEFC2 and GRiA2* are both ASD-risk genes which play a role in synaptic plasticity via AMPA receptors (AMPAR) trafficking in the synapse (Carmicheal et al., 2018). *MEFC2* expression regulates the activation and expression of *GRiA2*, which is responsible for the localization and trafficking of AMPARs in the postsynaptic membrane, which is an essential process for synaptic plasticity (Carmicheal et al., 2018, Chater et al., 2014). Therefore, since there is a reduction in COX2-PGE_2 signalling in our mouse model, which coincides with a downregulation and altered function of these two genes, it is probable that we would observe a decrease in synaptic connectivity in the same mice, via a decrease in AMPAR trafficking.

Furthermore, altered COX-2 signalling contributes to altered MAPK pathway signalling as well. For example, COX2-PGE_2 signalling plays a crucial role in ERK phosphorylation, which has downstream effects on long-term potentiation (Kelleher et al., 2005). As previously discussed, there are several genes which were identified in study 1 to be associated with MAPK signalling, in particular the ERK subclass of this pathway (Poddar et al., 2019, Chen et al., 2017). *MEFC2 and GRiA2* have been linked to the p38 and ERK subclasses of the MAPK signalling pathway (Poddar et al., 2019, Chen et al., 2017). This pathway plays a role in synaptic plasticity and long-term potentiation, where abnormalities in the signalling pathway can result in abnormal synaptic plasticity and an altered degree of long-term potentiation (Kelleher et al., 2005). PGE_2 signalling can alter this pathway, and it was also observed that deficiencies in PGE_2 also significantly downregulate the expression of *MEFC2 and GRiA2*. This suggest that there may be the possibility that PGE_2 alters MAPK expression and synaptic plasticity through the dysregulation of *MEFC2 and GRiA2*.

5.1.8 Synaptic Plasticity and ASD

There has been a lot of research connecting ASD and synaptic plasticity. There has been ample evidence to indicate that there are abnormalities in synaptic plasticity in individuals with ASD (Kelleher and Bear, 2005). Due to this, many researchers note that abnormal levels of synaptic plasticity is an underlying pathology for ASD (Kelleher and Bear, 2005). In study 1, we identified 6 genes which were a part of the synaptic plasticity group, *Fmr1, cyfp1, Grin2b, EiF4e, GRiA2, and MEF2.* All of these genes were identified to be ASD-risk genes, indicating that dysregulations in their expression have been tied to ASD.

Alterations in synaptic plasticity have been associated with many phenotypic characteristics of ASD. For instance, many deficits in ASD such as, motor coordination, intellectual ability, social behaviour and language, can be associated with reduced synaptic plasticity and long-term potentiation, in specific brain regions (Gilbert et al., 2014). These deficiencies in synaptic plasticity result in disruptions to many neuronal circuits which are involved in language, motor and social behaviours associated with ASD (Gilbert et al., 2014). A study in our lab, using the COX-2 KI mouse model, exhibited that the mice showed increased anxiety, hyperactivity, motor defects, and decreased sociability, all of which are associated with ASD behaviours and disruptions in synaptic plasticity (Wong et al., 2017). Potentially the genes identified in study 1 to be associated with synaptic plasticity may have played a role in these ASD-like behaviours.

Furthermore, synaptic plasticity is also associated with ASD via genetic factors as well. There has been evidence that ASD-linked gene mutations lead to abnormal gene transcription

and protein synthesis associated with altered neuronal circuitry and deficits in synaptic plasticity (Guang et al., 2018). For instance, studies have indicated that in brain regions which exhibit synaptic deficiencies, there are also deficits in *Fragile X mental retardation protein* (*FMRP)* expression (Sidorov et al., 2013). This also relates to evidence which connects increased synaptic loss with Fragile X syndrome (FXS) (Sidorov et al., 2013). FMRP is responsible for the translation of several synaptic proteins, needed for synaptic strength (Osterweil et al., 2010). However, FMRP is silenced in FXS, which leads to reduced synaptic strength and an overall loss of synaptic connections (Qin et al., 2005). Additionally, abnormal *TSC1 and TSC2* expression has been identified in synaptic dysfunction and cognitive impairments which are similar to those observed in ASD individuals (Kirschestein et al., 2012). Dysregulations in *TSC1 and TSC2* lead to downstream effects on mTOR signalling, which play a role on neurotransmission and dendritic spine formation (Talos et al., 2008). It has been observed that dysregulation in these two genes leads to abnormal trafficking of AMPARs, which as discussed above, is essential for synaptic connections and learning and memory (Talos et al., 2008, Bateup et al., 2011). Furthermore, evidence has suggested that dysregulations in *TSC1, TSC2, and FMRP*, alter AMPAR function in neurons, which influences the synaptic connectivity in a manner commonly observed in individuals with ASD (Auberach et al., 2011). This indicates that deficits associated with synaptic plasticity observed in individuals with ASD, can be driven by abnormal gene expression. Therefore, the information presented in study 1 could provide insight into how some of the synaptic plasticity genes may contribute to ASD.

5.2 Study 2: The effects of PGE_2 on the production of superoxide anion, in neuronal cells, as an indication of the effects of PGE_2 on ROS production.

5.2.1 Overview of Results

As mentioned previously, study 2 was conducted in differentiated neuroectodermal (NE-4C) stem cells (Davidson et al., 2014, Wong et al, 2014). The differentiated NE-4C cells were used to determine if the treatment of 1 µM and 10µM PGE_2 would increase the amount of superoxide anion produced in the mitochondria and nitric oxide produced in the cytosol, in a dose-dependent manner. Time-lapse fluorescent microscopy was used and the concentrations and kinetic rate of the amount of superoxide and nitric oxide produced in the cells was determined.

The results gathered from study 2 demonstrate that PGE_2 does indeed alter oxidative stress in neuronal cells. Previous literature has indicated that altered levels of oxidative stress in many cells, including neuronal cells, has an impact on the production of PGE_2 within these cells (Black et al., 2012, Fang et al., 2015, Wang et al., 2004). Additionally, there have been studies which indicate that increased levels of PGE_2 can also influence the amount of oxidative stress generated in motor neurons (Kosuge et al., 2020). The results from study 2, provide evidence that there is a positive feedback loop in which altered levels of PGE_2 can also influence the production of reactive oxygen species in neuronal cells. It was observed that increasing PGE_2 concentration in neuronal cells, leads to increased production of superoxide anion and nitric oxide. This production of reactive oxygen species and reactive nitrogen species is an indicator of oxidative stress within the cell. Therefore, it was observed that by increasing PGE_2 concentration within neuronal cells, there was an increase in oxidative stress in the cells as well. This suggests

that PGE_2 and oxidative stress can influence the production of each other, indicating a feedback loop.

Interestingly, we also noted that two different concentrations of PGE_2 can have significantly different effects on the concentration and ratio of reactive oxygen and nitrogen species produced within neuronal cells. It was observed that there is a significant difference in the amount of superoxide anion and nitric oxide produced within neuronal cells, when the concentration of PGE_2 that is added to the cells is increased from 1µM to 10 µM. This indicates that there is a dose-dependent effect which PGE_2 has on reactive oxygen and reactive nitrogen species production. Therefore, PGE_2 acts in a dose-dependent manner in respect to its contribution to oxidative stress within neuronal cells.

The use of NE-4C cells is a suitable model for these experiments and provides additional information about the connection between oxidative stress and the developing brain, since this cell model shares many similarities to the developing brain, such as the cell's ability to proliferate, migrate and differentiate into neurons. Oxidative stress and altered PGE_2 signalling can have detrimental effects on the brain, especially during fetal development and childhood. Many of these effects have been associated with many neurological disorders.

5.2.2 Oxidative stress and the Brain

As previously discussed, PGE_2 is the major lipid signalling molecule in the brain. This explains why PGE_2 signalling can have many implications in healthy brain development. The brain is also the area of the body which is known to have the least amount of antioxidants, making it increasingly more susceptible to the production of reactive oxygen and nitrogen species, which in turn leads to the brain being the most susceptible organ to oxidative stress

(Cole et al, 2016). Furthermore, neurons are the cell type which consume the most energy within the body and also have the lowest antioxidant capacity (Cobley et al., 2018). This combination leads to neurons being highly susceptible to oxidative stress. The use of NE-4C cells in study 2 supports these findings since the neuronal cells readily produced increased levels of superoxide and nitric oxide during the experiments.

Furthermore, children have been observed to be more susceptible to oxidative stress compared to adults (Nasca et al., 2010, Avlonini et al., 2017). Children are known to have lower levels of antioxidants such as glutathione, compared to their adult counterparts (Avlonini et al., 2017). Throughout conception and infancy, there are extremely low levels of antioxidants in the brain of the offspring, which indicates that during this period of brain development, even low doses of reactive oxygen and nitrogen species can take a lasting toll on the brain, well after birth (Nasca et al., 2010). It has been observed in newborns that there are low levels of antioxidants in the brain, along with high levels of reactive oxygen species (Milan et al., 2018). Oxidative stress has been noted to lead to many negative effects on cells, such as lipid peroxidation, DNA and protein oxidation, abnormal immune responses, and apoptosis (Utarra et al., 2009). Additionally, oxidative stress has been tied to many neurological disorders such as ASD, AZD and PD (Patel et al., 2016, Utarra et al., 2009). Additionally, there is a growing field of research which is investigating the possibility of treating newborns with antioxidant supplements, in order to reduce the risk of developing a disorder which is connected to increased levels of oxidative stress, such as ASD (Patel et al., 2016, Utarra et al., 2009).

There has been an abundance of literature discussing how oxidative stress can occur in the brain. The results observed in study 2, along with other research from our lab indicates that PGE_2 signalling could play a role in oxidative stress generation in the brain. As mentioned

above, PGE_2 plays a crucial role in healthy brain development. The data from study 2 suggest that PGE_2 can also generate increased levels of oxidative stress, in a dose-dependent manner in neuronal cells. There are many factors during development which can alter PGE_2 expression in an abnormal manner. This alteration in PGE_2 concentration can therefore lead to altered levels of oxidative stress, through reactive oxygen and nitrogen specie generation, especially in neuronal cells. This increased level of oxidative stress can have detrimental effects on the brain and lead to many neurological disorders, including ASD (Patel et al., 2016).

5.2.3 PGE_2, Oxidative Stress and ASD

As previously mentioned, there has been an abundance of literature which has connected abnormalities in PGE_2 with ASD. For instance, PGE_2 has been observed to influence genetic mutations which play a role in ASD. Additionally, abnormalities in the COX2-PGE_2 signaling pathway can be due to genetic factors and environmental factors which can also lead to ASD-like behaviours (Yoo et al., 2009, Sethi et al., 2019). There are many environmental factors which can influence PGE_2 levels in the developing brain, and thus lead to ASD. Furthermore, PGE_2 plays crucial roles in dendritic spine formation, learning and memory and synaptic plasticity, all of which are also associated with ASD (Chen et al., 02, Chen and Brazan, 2005, Burks et al., 2007).

There is also evidence that oxidative stress plays a crucial role in many factors which can lead to ASD. Many researchers suggest that oxidative stress is an integrated aspect of ASD neurobiology (Saidel-Sulkowska et al., 2008). This is due to the fact that ASD neurobiology shows increased levels of reactive oxygen species in the brain, which is an indication of excess

levels of oxidative stress (Palmeri et al., 2010). Evidence suggest that children are vulnerable to ASD even after birth, due to the continued imbalance of glutathione antioxidant reserves children possess (Yasuda et al., 2015) This is one of the reasons why many people suggest that treating newborns with antioxidants will drastically reduce the risk of ASD (Evans et al., 2008, Yasuda et al., 2015).

Many studies also indicate that there remains a high level of oxidative stress in individuals with ASD throughout their lives. For example, blood samples of individuals with ASD indicate significantly high levels of oxidative stress biomarkers compared to a control group (Rose et al., 2012). Additionally, in brain tissue samples of individuals with ASD, the brain regions associated with speech, motor function, social behaviour and sensory perception, showed significantly higher biomarkers of oxidative stress compared to control groups (Muratore et al., 2013). This demonstrates that if we can find a way of using these biomarkers for diagnostic purposes, we can use oxidative stress levels as a means to diagnose individuals with ASD.

The connection between PGE_2 and ASD, as well as the connection between oxidative stress and ASD, has been highlighted. It is evident that ASD is connected to both PGE_2 and oxidative stress, which can both be altered within the brain. Using the results from study 2, we can see that there is a direct link between PGE_2 and oxidative stress, in the form of a positive feedback loop. The brain, as well as its neurons, are susceptible to alterations in PGE_2 signalling, which can in turn lead to the generation of reactive oxygen and nitrogen species. This results in oxidative stress levels in the brain changing, which can have a lasting impact of the brain, leading to ASD (Rose et al., 2012). PGE_2 and Oxidative stress both lead to similar effects within cells. For example, they both can influence gene and protein expression. PGE_2 and oxidative

stress can also lead to disruptions in cell function, such as disruptions in organelle function and complications in cell survival.

5.2.4 Oxidative stress, mitochondrial function and ASD

In study 2, altered concentrations of PGE_2 led to the production of superoxide anion in the mitochondria. This indicates that PGE_2 can directly influence the mitochondria. Increased concentrations of PGE_2 led to the significant increase in superoxide production in a dose-dependent manner. This further indicates that PGE_2 can influence mitochondrial function.

Oxidative stress has also been known to influence mitochondrial function. Literature has indicated that there is a co-existence between oxidative stress and mitochondrial dysfunction (Chakrabati et al., 2011). Mitochondrial dysfunction has also been associated with ASD (Hass er al., 2010). There have been many studies which highlight that individuals with ASD exhibit, decreased electron transport chain function, dysregulations in mitochondrial genes, as well as increased signs of oxidative stress (Wallace et al., 2013, Naviaux et al., 2005).

There has been little research done explaining how PGE_2 can alter mitochondrial function. There is evidence to suggest that altered levels of PGE_2, coincide with altered mitochondrial function, such as decrease electron transport chain function, and increased electron transport chain leaks, which in turn leads to increased superoxide production (Bacon et al., 2013). However, there has not been much literature directly connecting PGE_2, oxidative stress and mitochondrial function, in neuronal cells.

The results in study 2, are the first to illustrate that PGE_2 can directly influence oxidative stress production by acting on the mitochondria. The superoxide anion detected using MitoSox Red is generated due to leaks in the electron transport chain, which is also an indication of

mitochondrial dysfunction. Since PGE_2 treated cells showed a significant increase in superoxide production, compared to untreated cells, there is strong evidence that increased levels of PGE_2 is one of the contributing factors to mitochondrial dysfunction in neuronal cells. Furthermore, since PGE_2 increased superoxide production in a dose-dependent manner, it is expected that PGE_2 can alter mitochondrial dysfunction in a dose-dependent manner as well. Therefore, this demonstrates that PGE_2 can influence oxidative stress through its effects on the mitochondria of neuronal cells. These findings offer new insight into a specific mechanism by which PGE_2 can influence oxidative stress in neurons.

5.3 Conclusion

5.3.1 Significance of findings and future studies

Based on the findings discussed above, it is evident that certain factors from study 1 and study 2 should be further investigated. In both study 1 and study 2 there was an association with the results and an increased risk of mitochondrial dysfunction. Study 1 illustrated that a lack of PGE_2 led to gene dysregulations in mice, which are also characteristic of mitochondrial dysfunction. However, study 2 demonstrated that adding increased concentrations of PGE_2 to neuronal cells led to increased superoxide anion production, which is characteristic of mitochondrial dysfunction. Both of these studies show that increased and decreased levels of PGE_2 can lead to devastating effects on the mitochondria. These mitochondrial effects should be further investigated, so that the degree of mitochondrial dysfunction caused by altered PGE_2 concentrations can be understood. For example, studies investigating how 1 µM and 10µM PGE_2 can alter mitochondrial function differently, should be conducted. This can be done using dyes specific to the mitochondria which monitor the organelle's function. Additionally, mitochondrial dysfunction biomarkers found in blood or brain tissue can be used on the KO mice to investigate if there are signs associated with mitochondrial dysfunction in these animals as well. This will allow for more conclusions to be drawn about the connection to gene dysregulations and mitochondrial dysfunction in our mouse model.

In study 2 we investigated the effects of PGE_2 on oxidative stress in a cell model. The next step for this study would be to see if similar patterns are observed in an animal model. Our lab has two well-established models which can be used to determine how COX2-PGE_2 signalling can lead to oxidative stress in animals. Using our Knockin and Knock-out models and oxidative

stress biomarkers specific to brain tissue, we can examine how varying degrees of COX2-PGE_2 signalling can influence oxidative stress in the brain.

Additionally, in study 1 gene expression for several ASD-risk genes could be conducted. There are several ASD-risk genes which are associated with oxidative stress that can be investigated using PCR. The presence and expression of the genes should be determined to see if the increased production of ROS and RNS can contribute to altered gene expression in neuronal cells. By investigating ASD-risk genes, we can find a possible mechanism by which PGE_2 and oxidative stress contribute to ASD. Furthermore, we could possibly investigate ASD-risk genes from study 2, such as *MEFC2, GRiA2, TSC2, and TGFBR2*. This could provide further insight into how PGE_2 and oxidative stress can contribute to ASD.

Additionally, study 1 discusses the gene dysregulations in KO mice which were associated with three different functional groups, mitochondrial function, inflammatory responses, and synaptic plasticity. To further investigate if these genes play a role in these specific functions in our model system, experiments can be conducted to determine if mitochondrial function, inflammatory responses and synaptic plasticity are altered in KO mice compared to WT mice. First, real-time PCR can be used to confirm the differential expression of each gene gathered from the microarray analysis. Next, biomarkers can be used in brain slices or blood to test for several abnormalities in mitochondrial function and inflammatory responses in our COX-2 KO animals. For instance, identifying oxidized proteins in brain tissue or disruptions in the ETC complexes, can be an indication of mitochondrial disruptions. There has been literature indicating that there are oxidized mitochondrial DNA and proteins in individuals with ASD (Tang et al., 2013). Also, disruptions in the ETC complexes such as complex I and III have been observed in children with ASD and associated with increased superoxide production

(Cillero-Pastor, et al., 2008, Zhang et al., 2018). Additionally, conducting western blot analysis to identify various pro-inflammatory cytokines can be an indication of amplified inflammatory responses. As mentioned previously, pro-inflammatory cytokines like interleukin-6 and interleukin-1ß have been observed to be overly activated in children with ASD compared to controls (Minami et al., 2001, Mariyama et al., 2005). Finally, electrophysiology can be used to evaluate synaptic connectivity in the brain slices of our animals to assess how synaptic circuitry is altered in COX-2 KO animals. There has been increasing research which has found a link between decreased synaptic connectivity and ASD (Chen et al., 2002, Kelleher and Bear, 2005, Rodriguez et al., 2011).

5.3.2 Limitations

The data collected for the H_2O_2 induced superoxide plot illustrated problematic superoxide production (Figure 9). This is evident due to the initial rise in superoxide production, then plateau, then rise once again. This is indicative of a potential technical error within the protocol that could be potentially improved. The abnormal trend observed with this treatment could result from the MitoSox Red not being evenly dispersed throughout the plate or some cells still being in neurosphere clusters.

To improve future studies, several procedural elements could be optimized. First, cells can be treated with dye first, and then placed on a slow-paced shaker, to help distribute the dye throughout the plate. Additionally, manually removing any remaining neurospheres before each experiment could increase the number of individual cells. Lastly, since the trends observed for

the H_2O_2 treated cells were the most atypical, a better positive control could be used. For example, Antimycin A can induce mitochondrial production of superoxide, which could also be detected by MitoSox Red and is more stable (Parath et al., 2007, Wang et al., 2015).

5.3.3 Conclusion

In summary, the data depicted in studies 1 and 2 illustrate that abnormal COX2-PGE_2 signalling can have implications at the genetic and cellular level, similar to abnormalities observed in individuals with ASD. Interestingly, it was observed that increases and decreases in PGE_2 can both have implications on the mitochondria. There has been ample evidence for mitochondrial dysfunction in ASD individuals (Tang et al, 2013, Sanin et al, 2018,). Additionally, mitochondrial dysfunction has been associated with increased pro-inflammation and abnormalities in synaptic connectivity (Islam, 2016, Sanin et al., 2018). This data provides some insight into the link between abnormal COX2-PGE_2 signalling and abnormal mitochondrial function in ASD.

Chapter 6-Bibliography

Akyol, Maria G. “Nitric Oxide and Peripheral Neurotransmission in the Lung.” Nitric Oxide and Free Radicals in Peripheral Neurotransmission, 2004, pp. 138–159., doi:10.1007/978-1 4612-13284_6.

Allely, C. S. “ Pain sensitivity and observer perception of pain in individuals with autistic spectrum disorder Pain Sensitivity and Observer Perception of Pain in Individuals with Autistic Spectrum Disorder”. Sci. World J. 2013, (2013).

Amateau, S. K. & McCarthy, M. M. Induction of PGE2 by estradiol mediates developmental masculinization of sex behavior. Nat. Neurosci. 7, 643–650 (2004).

Arcoleo, Vincenzo, et al. “The Effect of Nitric Oxide on Cytokine-Induced Release of PGE2by Human Cultured Astroglial Cells.” British Journal of Pharmacology, vol. 124, no. 4, 1995, pp. 742–746., doi:10.1038/sj.bjp.0701852

Auerbach, B. D., Osterweil, E. K. & Bear, M. F. Mutations causing syndromic autism define an axis of synaptic pathophysiology. Nature 480, 63–68 (2011).

Bandim, J. M., Ventura, L. O., Miller, M. T., Almeida, H. C. & Santos Costa, A. E. AUTISM AND MÖBIUS SEQUENCE An exploratory study of children in northeastern Brazil. Arq Neuropsiquiatr 61, (2003).

Bateup, H. S., Takasaki, K. T., Saulnier, J. L., Denefrio, C. L. & Sabatini, B. L. Loss of Tsc1 in vivo impairs hippocampal mGluR-LTD and increases excitatory synaptic function. J. Neurosci. 31, 8862–8869 (2011).

Bell, J. G. et al. Essential fatty acids and phospholipase A2 in autistic spectrum disorders. In Prostaglandins Leukotrienes and Essential Fatty Acids 71, 201–204 (Prostaglandins Leukot Essent Fatty Acids, 2004).

Bell, John Gordon, et al. “The Fatty Acid Compositions of Erythrocyte and Plasma Polar Lipids in Children with Autism, Developmental Delay or Typically Developing Controls and the Effect of Fish Oil Intake.” British Journal of Nutrition, vol. 103, no. 8, 2004, pp. 1160 1167., doi:10.1017/s0007114509992881.

Bigarella, C. L., et al. “Stem Cells and the Impact of ROS Signaling.” Development, vol. 141, no. 22, 2014, pp. 4206–4218., doi:10.1242/dev.107086.

Black, Micheal P., et al. “Keratinocyte Prostaglandin Synthesis Is Enhanced by IL-1.” Journal of Investigative Dermatology, vol. 94, no. 1, 2011, pp. 43–46., doi:10.1111/1523 1747.ep12873337.

Blackwell, K. A., Raisz, L. G. & Pilbeam, C. C. Prostaglandins in bone: Bad cop, good cop? Trends in Endocrinology and Metabolism 21, 294–301 (2010).

Blanco, Berta, et al. “Mitochondrial Dysfunction Activates Cyclooxygenase 2 Expression in Cultured Normal Human Chondrocytes.” Arthritis & Rheumatism, vol. 58, no. 8, 2008, pp. 2409–2419., doi:10.1002/art.23644.

Burlacu, A. Regulation of apoptosis by Bcl-2 family proteins. Journal of Cellular and Molecular Medicine 7, 249–257 (2003).

Carmichael, R. E., Wilkinson, K. A., Craig, T. J., Ashby, M. C. & Henley, J. M. MEF2A regulates mGluR dependent AMPA receptor trafficking independently of Arc/Arg3.1. Sci. Rep. 8, (2018).

Chahum, Rafiq. “Significance of Brain Tissue Oxygenation and the Arachidonic Acid Cascade in Stroke.” Antioxidants & Redox Signaling, vol. 14, no. 10, 2016, pp. 1889–1903., doi:10.1089/ars.2010.3474.

Chater, T. E. & Goda, Y. The role of AMPA receptors in postsynaptic mechanisms of synaptic plasticity. Frontiers in Cellular Neuroscience 8, (2014).

Chauhan, A. et al. Brain region-specific deficit in mitochondrial electron transport chain complexes in children with autism. J. Neurochem. 117, 209–220 (2011).

Chen, C., Magee, J. C. & Bazan, N. G. Cyclooxygenase-2 Regulates Prostaglandin E 2 Signaling in Hippocampal Long-Term Synaptic Plasticity. J. Neurophysiol. 87, 2851–2857 (2002).

Chen, J. L., Leader, G., Sung, C. & Leahy, M. Trends in Employment for Individuals with AutismSpectrum Disorder: a Review of the Research Literature. Review Journal of Autism and Developmental Disorders 2, 115–127 (2015).

Chen, R., Jiao, Y. & Herskovits, E. H. Structural MRI in autism spectrum disorder. Pediatr. Res. 69, 63R (2011).

Cheng, Micheal, et al. “Frontiers in Nitric Oxide and Redox Signaling.” Nitric Oxide, vol. 25, no. 2, 2011, pp. 57–58., doi:10.1016/j.niox.2011.05.002.

Cheng, N., Rho, J. M. & Masino, S. A. Metabolic dysfunction underlying autism spectrum disorder and potential treatment approaches. Frontiers in Molecular Neuroscience 10, 34 (2017).

Chez, M. G., Dowling, T., Patel, P. B., Khanna, P. & Kominsky, M. Elevation of Tumor Necrosis Factor Alpha in Cerebrospinal Fluid of Autistic Children. Pediatr. Neurol. 36, 361–365 (2010).

Cina, Cima, et al. “Expression of Connexins in Embryonic Mouse Neocortical Development.” The Journal of Comparative Neurology, vol. 504, no. 3, 2007, pp. 298–313., doi:10.1002/cne.21426.

Cobley, James Nathan, et al. “13 Reasons Why the Brain Is Susceptible to Oxidative Stress.” Redox Biology, vol. 15, 2018, pp. 490–503., doi:10.1016/j.redox.2018.01.008.

Cole, Toby B., et al. “Sex and Genetic Differences in the Effects of Acute Diesel Exhaust Exposure on Inflammation and Oxidative Stress in Mouse Brain.” Toxicology, vol. 374, 2016, pp. 1–9., doi:10.1016/j.tox.2016.11.010.

Corrado, David E., et al. “Mitochondrial Membrane Potential Regulates Nuclear Gene Expression in Macrophages Exposed to Prostaglandin E2.” Immunity, vol. 49, no. 6, 2018, doi:10.1016/j.immuni.2018.10.011.

Costa, Eduardo D., et al. “Neuronal Nitric Oxide Synthase in Vascular Physiology and Diseases.” Frontiers in Physiology, vol. 7, 2016, doi:10.3389/fphys.2016.00206.

Dean, G. M. Neuropharmacological mechanisms underlying the effect of medial preoptic area dopamine in the sexual behaviour of female rats. (2017).

Dean, S. L., Knutson, J. F., Krebs-Kraft, D. L. & Mccarthy, M. M. Prostaglandin E2 is an endogenous modulator of cerebellar development and complex behavior during a sensitive postnatal period. Eur. J. Neurosci. 35, 1218–1229 (2012).

Devitt, N., Gallagher, L. & Reilly, R. Autism Spectrum Disorder (ASD) and Fragile X Syndrome (FXS): Two Overlapping Disorders Reviewed through Electroencephalography—What Can be Interpreted from the Available Information? Brain Sci. 5, 92–117 (2015).

Dominko, Kristina, and Domagoj Đikić. “Glutathionylation: a Regulatory Role of Glutathione in Physiological Processes.” Archives of Industrial Hygiene and Toxicology, vol. 69, no. 1, 2018, pp. 1–24., doi:10.2478/aiht-2018-69-2966.

Dröse, Stefan, and Ulrich Brandt. “Molecular Mechanisms of Superoxide Production by the Mitochondrial Respiratory Chain.” Advances in Experimental Medicine and Biology Mitochondrial Oxidative Phosphorylation, 2012, pp. 145–169., doi:10.1007/978-1-4614-35730_6.

Esplugues, Juan V. “NO as a Signalling Molecule in the Nervous System.” British Journal of Pharmacology, vol. 135, no. 5, 2002, pp. 1079–1095., doi:10.1038/sj.bjp.0704569.

Evans, T. A. et al. The autistic phenotype exhibits a remarkably localized modification of brain protein by products of free radical-induced lipid oxidation. Am. J. Biochem. Biotechnol. 4, 61–72 (2008).

Fang, Lanlan, et al. “Nitric Oxide and CGMP Induce COX-2 Expression and PGE2Production in HumanGranulosa Cells Through CREB Signaling Pathway.” The Journal of Clinical Endocrinology & Metabolism, vol. 100, no. 2, 2015, doi:10.1210/jc.2014-2886.

Farooqui, A. A., Horrocks, L. A. & Farooqui, T. Modulation of inflammation in brain: A matter of fat. Journal of Neurochemistry 101, 577–599 (2007).

Farooqui, Akhlaq A., et al. “Modulation of Inflammation in Brain: a Matter of Fat.” Journal of Neurochemistry, vol. 101, no. 3, 2007, pp. 577–599., doi:10.1111/j.1471 4159.2006.04371.x.

Forrester, Michael T., et al. “Detection of Protein S-Nitrosylation with the Biotin-Switch Technique.” Free Radical Biology and Medicine, vol. 46, no. 2, 2009, pp. 119–126.,

Forstermann, U., and W. C. Sessa. “Nitric Oxide Synthases: Regulation and Function.” European Heart Journal, vol. 33, no. 7, 2002, pp. 829–837., doi:10.1093/eurheartj/ehr304.

Furuyashiki, T. & Narumiya, S. Fever, inflammation, pain and beyond: prostanoid receptor research during these 25 years. FASEB J. 25, 813–818 (2011).

Gaigalas, A.k., et al. “Quantitating Fluorescence Intensity from Fluorophore: Assignment of MESF Values.” Journal of Research of the National Institute of Standards and Technology, vol. 110, no. 2, 2005, p. 101., doi:10.6028/jres.110.010.

Ganesan, H. et al. mTOR signalling pathway - A root cause for idiopathic autism? BMB Rep. 52, 424 433 (2019).

Gersting, J. A., Schaub, C. E. & Wood, C. E. Development of prostaglandin endoperoxide synthase expression in the ovine fetal central nervous system and pituitary. Gene Expr. Patterns 9, 603611 (2009).

Ghanian, Zahra, Girija Ganesh Konduri, Said Halim Audi, Amadou K.S. Camara, and Mahsa Ranji. “Quantitative Optical Measurement of Mitochondrial Superoxide Dynamics in Pulmonary Artery Endothelial Cells.” *Journal of Innovative Optical Health Sciences* 11, no. 1 (January 1, 2018). https://doi.org/10.1142/S1793545817500183.

Gilbert, J. R. & Zarate, C. A. Electrophysiological biomarkers of antidepressant response to ketamine in treatment-resistant depression: Gamma power and long-term potentiation. Pharmacology Biochemistry and Behavior 189, (2014).

Gilbert, K. M. et al. Oxidative Stress Challenge Uncovers Trichloroacetaldehyde Hydrate Induced Mitoplasticity in Autistic and Control Lymphoblastoid Cell Lines. Sci. Rep. 7, (2014).

Giulivi, C. et al. Mitochondrial dysfunction in autism. JAMA - J. Am. Med. Assoc. 304, 2389 2396 (2010).

Goh, S., Dong, Z., Zhang, Y., DiMauro, S. & Peterson, B. S. Mitochondrial dysfunction as a neurobiological subtype of autism spectrum disorder: Evidence from brain imaging. JAMA Psychiatry 71, 665 671 (2014).

Gordon Bell, J. et al. The fatty acid compositions of erythrocyte and plasma polar lipids in children with autism, developmental delay or typically developing controls and the effect of fish oil intake. Br. J. Nutr. 103, 1160–1167 (2010).

Guang, S. et al. Synaptopathology involved in autism spectrum disorder. Frontiers in Cellular Neuroscience 12, 470 (2018).

Haas, R. H. Autism and mitochondrial disease. Dev. Disabil. Res. Rev. 16, 144–153 (2010).

Hatae, N., Yamaoka, K., Sugimoto, Y., Negishi, M. & Ichikawa, A. Augmentation of receptor mediated adenylyl cyclase activity by Gi-coupled prostaglandin receptor subtype EP3 in a Gβγ subunit independent manner. Biochem. Biophys. Res. Commun. 290, 162–168 (2002).

Hewett, S. J. et al. Spontaneous glutamatergic synaptic activity regulates constitutive COX-2 expression in neurons: Opposing roles for the transcription factors CREB (cAMP response element binding) protein and Sp1 (stimulatory protein-1). J. Biol. Chem. 291, 27279–27288 (2016).

Hewett, S. J., Bell, S. C. & Hewett, J. A. Contributions of cyclooxygenase-2 to neuroplasticity and neuropathology of the central nervous system. Pharmacology and Therapeutics 112, 335–357 (2006).

Hu, J. et al. WD40 protein FBW5 promotes ubiquitination of tumor suppressor TSC2 by DDB1 CUL4 ROC1 ligase. Genes Dev. 22, 866–871 (2014).

Islam, Md. Torequl. "Oxidative Stress and Mitochondrial Dysfunction-Linked Neurodegenerative Disorders." Neurological Research, vol. 39, no. 1, 2016, pp. 73 82., doi:10.1080/01616412.2016.1251711.

Jason, J. L. et al. TGF-β activity protects against inflammatory aortic aneurysm progression and complications in angiotensin II-infused mice. J. Clin. Invest. 120, 422–432 (2010).

Jory, J. Abnormal fatty acids in Canadian children with autism. Nutrition 32, 474–477 (2016).

Jung, Pang-Pin, et al. "Activin A Upregulates PTGS2 Expression and Increases PGE2 Production in HumanGranulosa-Lutein Cells." Reproduction, vol. 152, no. 6, 2013, pp. 655–664., doi:10.1530/rep-16 0262.

Kaufmann, W. E., Worley, P. F., Pegg, J., Bremer, M. & Isakson, P. COX-2, a synaptically induced enzyme, is expressed by excitatory neurons at postsynaptic sites in rat cerebral cortex. Proc. Natl. Acad. Sci. U. S. A. 93, 2317–2321 (1996).

Kelleher, R. J., Govindarajan, A., Jung, H. Y., Kang, H. & Tonegawa, S. Translational control by MAPK signaling in long-term synaptic plasticity and memory. Cell 116, 467–479 (2004).

Kirkby, N. S. et al. Cyclooxygenase-1, not cyclooxygenase-2, is responsible for physiological production of prostacyclin in the cardiovascular system. Proc. Natl. Acad. Sci. U. S. A. 109, 17597–17602 (2012).

Kosuge, Yasuhiro, et al. "Generation of Cellular Reactive Oxygen Species by Activation of the EP2 Receptor Contributes to Prostaglandin E2-Induced Cytotoxicity in Motor Neuron Like NSC-34 Cells." Oxidative Medicine and Cellular Longevity, vol. 2020, 2020, pp. 1–14., doi:10.1155/2020/6101838.

Kwan, G. et al. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. 28, 511 515 (2016).

Lawrence, Shoiw R. “Reactive Astrocyte COX2-PGE2 Production Inhibits Oligodendrocyte Maturation in Neonatal White Matter Injury.” Glia, vol. 65, no. 12, 2010, pp. 2024 2037., doi:10.1002/glia.23212.

Lee, Angus Y., et al. “NAIP-NLRC4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and IL-18 Release via Activation of Caspase-1 and -8.” Immunity, vol. 46, no. 4, 2017, pp. 649–659., doi:10.1016/j.immuni.2017.03.016.

Lee, J. H. et al. PGE2 Augments Inflammasome Activation and M1 Polarization in Macrophages Infected With Salmonella Typhimurium and Yersinia enterocolitica. Front. Microbiol. 9, (2018).

Lei, W. et al. Elevated COX-2 Expression Promotes Angiogenesis Through EGFR/p38 MAPK/Sp1 Dependent Signalling in Pancreatic Cancer. Sci. Rep. 7, (2017).

Licausi, F. & Hartman, N. W. Role of mTOR complexes in neurogenesis. International Journal of Molecular Sciences 19, (2018).

Loomis, W. F. & Laub, M. T. A molecular network that produces spontaneous oscillations in excitable cells of Dictyostelium. Mol. Biol. Cell 9, 3521–3532 (1998).

Lushchak, Volodymyr I. “Free Radicals, Reactive Oxygen Species, Oxidative Stress and Its Classification.” Chemico-Biological Interactions, vol. 224, 2014, pp. 164–175., doi:10.1016/j.cbi.2014.10.016.

Maldonado, E. N., Patnaik, J., Mullins, M. R. & Lemasters, J. J. Free tubulin modulates mitochondrial membrane potential in cancer cells. Cancer Res. 70, 10192–10201 (2010).

Mariyam, N., Yadegar, A. & Zali, M. R. A complex scenario of nonsteroidal anti-inflammatory drugs induced prostaglandin e2 production and gut microbiota alteration in clostridium difficile infected mice. mBio 11, (2020).

Masi, A. et al. Cytokine levels and associations with symptom severity in male and female children with autism spectrum disorder. Mol. Autism 8, 63 (2007).

Meeys, H. The Action of Prostaglandins on Ion Channels. Curr. Neuropharmacol. 4, 41–57 (2005).

Meguid, Nagwa A, et al. “Expression of Reactive Oxygen Species–Related Transcripts in Egyptian Children With Autism.” Biomarker Insights, vol. 12, 2017, p. 117727191769103., doi:10.1177/1177271917691035.

Meyers, A., Weiskittel, T. M. & Dalhaimer, P. Lipid Droplets: Formation to Breakdown. Lipids 52, 465475 (2017).

Minami, T. et al. Characterization of EP receptor subtypes responsible for prostaglandin E2 induced pain responses by use of EP1 and EP3 receptor knockout mice. Br. J. Pharmacol. 133, 438–444 (2001).

Muratore, F., Bellani, M., Calderoni, S. & Brambilla, P. Brain anatomy of autism spectrum disorders I. Focus on corpus callosum. Epidemiology and Psychiatric Sciences 22, 217 221 (2013).

Naviaux, R. K. et al. Chronic treatment of mitochondrial disease patients with dichloroacetate. Mol. Genet. Metab. 83, 138–149 (2005).

Osterweil, E. K., Krueger, D. D., Reinhold, K. & Bear, M. F. Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. J.Neurosci. 30, 15616–15627 (2010).

Partha, Mohanraj Rajesh, György Haskó, Brian J. Hawkins, Muniswamy Madesh, and Pál Pacher. "Simultaneous Detection of Apoptosis and Mitochondrial Superoxide Production in Live Cells by Flow Cytometry and Confocal Microscopy." *Nature Protocols* 2, no. 9 (September 2007): 2295–2301. https://doi.org/10.1038/nprot.2007.327.

Palmieri, L. et al. Altered calcium homeostasis in autism-spectrum disorders: Evidence from biochemical and genetic studies of the mitochondrial aspartate/glutamate carrier AGC1. Mol. Psychiatry 15, 38–52 (2010).

Poddar, R. & Paul, S. Novel crosstalk between ERK MAPK and p38 MAPK leads to homocysteine NMDA receptor-mediated neuronal cell death. J. Neurochem. 124, 558 570 (2013).

Poddar, R., Chen, A., Winter, L., Rajagopal, S. & Paul, S. Role of AMPA receptors in homocysteine NMDA receptor-induced crosstalk between ERK and p38 MAPK. J. Neurochem. 142, 560–573 (2017).

Quan, Y. Q. et al. Protein expression profiling of the Drosophila fragile X mutant brain reveals up regulation of monoamine synthesis. Mol. Cell. Proteomics 4, 278–290 (2005).

Rai-Bhogal, Ravneet, et al. "Maternal Exposure to Prostaglandin E2 Modifies Expression of Wnt Genes in Mouse Brain – An Autism Connection." Biochemistry and Biophysics Reports, vol. 14, 2018, pp. 43–53., doi:10.1016/j.bbrep.2017.03.012.

Retton, V., et al. "Nitric Oxide Mediates Norepinephrine-Induced Prostaglandin E2 Release from the Hypothalamus." Proceedings of the National Academy of Sciences, vol. 89, no. 23, 1992, pp. 11543–11546., doi:10.1073/pnas.89.23.11543.

Robb, Ellen L., et al. "Control of Mitochondrial Superoxide Production by Reverse Electron Transport at Complex I." Journal of Biological Chemistry, vol. 293, no. 25, 2018, pp. 9869–9879., doi:10.1074/jbc.ra118.003647.

Robson, M. J. et al. P38a MAPK signaling drives pharmacologically reversible brain and gastrointestinal phenotypes in the SERT Ala56 mouse. Proc. Natl. Acad. Sci. U. S. A. 115, E10245–E10254 (2018).

Rodríguez-Moreno, A. & Mateos-Aparicio, P. The impact of studying brain plasticity. Frontiers in Cellular Neuroscience 13, 66 (2011).

Rose, S. et al. Evidence of oxidative damage and inflammation associated with low glutathione redox status in the autism brain. Transl. Psychiatry 2, (2012).

Rosina, E. et al. Disruption of mTOR and MAPK pathways correlates with severity in idiopathic autism.Transl. Psychiatry 9, (2019).

Rossignol, D. A. & Frye, R. E. Evidence linking oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism. Frontiers in Physiology 5 APR, (2014).

Saarinen, L. et al. Multiple components of PKA and TGF-β pathways are mutated in pseudomyxoma peritonei. PLoS One 12, (2017).

Sachs, D., Villarreal, C. F., Cunha, F. Q., Parada, C. A. & Ferreira, S. H. The role of PKA and PKCη pathways in prostaglandin E 2-mediated hypernociception. Br. J. Pharmacol. 156, 826–834 (2009).

Saha. Michelle et al. Transforming Growth Factor-β in Thoracic Aortic Aneurysms: Good, Bad, or Irrelevant? Journal of the American Heart Association 6, (2017).

Sangwon, Kim F. "The Nitric Oxide-Mediated Regulation of Prostaglandin Signaling in Medicine." Vitamins & Hormones Nitric Oxide, 2014, pp. 211–245., doi:10.1016/b978-0-12-800254 4.00009-x.

Sanin, D. E. et al. Mitochondrial Membrane Potential Regulates Nuclear Gene Expression in Macrophages Expose

Sapkota, G. P. The TGFβ-induced phosphorylation and activation of p38 mitogen-activated protein kinase is mediated by MAP3K4 and MAP3K10 but not TAK1. Open Biol. 3, (2013).

Saxton, R. A. & Sabatini, D. M. mTOR Signaling in Growth, Metabolism, and Disease. Cell 168, 960 976 (2017).

Schroen, B. et al. The microRNA-15 family inhibits the TGFβ-pathway in the heart. Cardiovasc. Res. 104, 61–71 (2016).

Schüler, L. et al. Pregnancy outcome after exposure to misoprostol in Brazil: A prospective, controlled study. Reprod. Toxicol. 13, 147–151 (1999).

Sethi, C. et al. Parent and professional perspectives on behavioral inflexibility in autism spectrum disorders: A qualitative study. Autism 23, 1236–1248 (2019).

Sidorov, M. S., Auerbach, B. D. & Bear, M. F. Fragile X mental retardation protein and synaptic plasticity. Molecular Brain 6, 15 (2013).

Sies, Helmut. "The Concept of Oxidative Stress After 30 Years." Biochemistry of Oxidative Stress, 2017, pp. 3–11., doi:10.1007/978-3-319-45865-6_1.

Talos, D. M. et al. The Interaction between Early Life Epilepsy and Autistic-Like Behavioral Consequences: A Role for the Mammalian Target of Rapamycin (mTOR) Pathway. PLoS One 7, e35885 (2012).

Tang, G. et al. Loss of mTOR-Dependent Macroautophagy Causes Autistic-like Synaptic Pruning Deficits Neuron 83, 1131–1143 (2014).

Tostes, M. H. F. de S. et al. Níveis plasmáticos de ácidos graxos e fosfolipase A2 em crianças com autismo. Trends Psychiatry Psychother. 35, 76–80 (2013).

Urbanska, A. S. et al. ZBP1 phosphorylation at serine 181 regulates its dendritic transport and the development of dendritic trees of hippocampal neurons. Sci. Rep. 7, (2017).

Uttara, B., Singh, A., Zamboni, P. & Mahajan, R. Oxidative Stress and Neurodegenerative Diseases: A Review of Upstream and Downstream Antioxidant Therapeutic Options. Curr. Neuropharmacol. 7, 65–74 (2009).

Varga, B. V. et al. Generation of diverse neuronal subtypes in cloned populations of stem-like cells. BMC Dev. Biol. 8, 89 (2008).

Vincent, Steven R., et al. "Nitric Oxide Signaling via CGMP-Stimulated Phosphodiesterase in Striatal Neurons." Synapse, vol. 64, no. 6, 2010, pp. 460–466., doi:10.1002/syn.20750.

Wallace, D. C. A mitochondrial bioenergetic etiology of disease. J. Clin. Invest. 123, 1405–1412 (2013).

Wang, Lingyan, Qi Duan, Tingting Wang, Mohamed Ahmed, Na Zhang, Yongmei Li, Lanying Li, and Xiaomei Yao. "Mitochondrial Respiratory Chain Inhibitors Involved in ROS Production Induced by Acute High Concentrations of Iodide and the Effects of Sod as a Protective Factor." *Oxidative Medicine and Cellular Longevity* 2015 (2015). https://doi.org/10.1155/2015/217670.

Xu, J. R. et al. Transducin beta-like gene FTL1 is essential for pathogenesis in fusarium graminearum. Eukaryot. Cell 8, 867–876 (2009).

Yamagata, K., Andreasson, K. I., Kaufmann, W. E., Barnes, C. A. & Worley, P. F. Expression of a mitogen inducible cyclooxygenase in brain neurons: Regulation by synaptic activity and glucocorticoids. Neuron 11, 371–386 (1993).

Yang, H. & Chen, C. Cyclooxygenase-2 in Synaptic Signaling. Curr. Pharm. Des. 14, 1443–1451 (2008).

Yehuda, R. Biological factors associated with susceptibility to posttraumatic stress disorder. Canadian Journal of Psychiatry 44, 34–39 (1999).

Yoo, H. J. et al. Association between PTGS2 polymorphism and autism spectrum disorders in Koreantrios. Neurosci. Res. 62, 66–69 (2008).

Zhang, Y., Wei, J. & Zhang, X. Clinical and genetic analysis of tuberous sclerosis complex associated renal angiomyolipoma in Chinese pedigrees. Oncol. Lett. 14, 7085–7090 (2017).

Zhuang, Y. et al. Mitochondrial oxidative stress activates COX-2/mPGES-1/PGE2 cascade induced by albumin in renal proximal tubular cells. Oncotarget 9, 9235–9245 (2018).