Astrocytic deficits in maintaining oxidative homeostasis within the Fragile X Syndrome Cortex

By

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A Thesis

Submitted to the School of Graduate Studies

In Partial Fulfillment of the Requirements

For the Degree

Master of Science

McMaster University

Hamilton, Ontario, Canada

MASTERS OF SCIENCE (2020) McMaster University

(Biology) Hamilton, Ontario

Title: Astrocytic deficits in maintaining oxidative homeostasis within the Fragile X Syndrome Cortex

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Number of pages: ix, 91

Abstract

Fragile X Syndrome (FXS) is caused by the instability of a CGG-repeated tract at the 5' end of the Fmr1 transcript. This instability causes silencing of the gene coding for FMRP. Higher levels of reactive oxygen species, lipid peroxidation, and protein oxidation within brain tissue have been found to be associated with the disease. These imbalances, along with altered levels of components of the glutathione system, provide evidence for increased oxidative stress. Astrocytes, glial cells within the brain, have many functions within neurodevelopment. Specifically, they regulate growth and synaptic contacts of neurons, regulate the level of excitability of synapses, and protect neurons at high levels of activity. To protect neurons from oxidative stress, astrocytes maintain oxidative homeostasis through their mitochondrial electron transport and antioxidant systems. This study examines the relationship between oxidative stress and FXS by assessing mitochondrial function and the antioxidant system of astrocytes. Using the *Fmr*1 knockout (KO) mouse model, mitochondrial respiration, and reactive oxygen species (ROS) production was analyzed in cultured cortical astrocytes. Astrocytes collected from male and female mice were analyzed under both normoxic and hypoxic conditions. In addition, western blots were conducted on both cortical tissue and cultured cortical astrocytes to determine potential differences in enzyme expression. Results indicate elevations of leak state respiration and ROS production in *Fmr*1 KO cultured cortical astrocytes alongside alterations in antioxidant and NADPH-oxidase expression. Characterization of mitochondrial function and the antioxidant system of astrocytes will be highly valuable to the understanding of glial roles during brain development and could provide future insight to direct clinically relevant studies of FXS and other neurodevelopment disorders.

Acknowledgements

During the past three years I have learned a lot of new things and made many new friends in both the departments of Biology and Anatomy. I want to express my deepest gratitude towards Dr. Angela Scott for all her guidance, support, friendship, and for her role in developing my skills as a scientific researcher. My thanks are also extended to Dr. Colin Nurse and Dr. Grant McClelland for sitting on my supervisory committee and providing guidance. Furthermore, I would like to thank Dr. Graham Scott for assisting in experimental design and Dr. Neal Dawson for his patience and training. Additionally, I would like to thank Dr. Mike O'Donnell for stepping in as the external examiner for my defence.

Coming into lab would not have been as enjoyable if not for the friends I made. I would like to thank Amanda Poxon, Kathryn Reynolds, Victoria Krasovska, David Shin, Alison Head, Aasritha Thotakura, Stephanie Scott, Eva Stefanova, and Matthew Napier for the friendship, advice, and encouragement they have provided. Specifically, I would like to acknowledge Alison's assistance in performing protein assays and western blotting experiments for SOD proteins present in Chapter 2 of this thesis. As well as Aasritha's assistance in tissue culture preparation and high resolution respirometry experiments for male and female comparisons made in Chapter 3.

I am grateful for my family for all the love, support, understanding, and encouragement they have provided me through my studies.

A very special thanks goes to Sabino Lops for his love, friendship, and support throughout this process. Thank you for seeing me through the bad times and making the good times even better.

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Abbreviations

ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
APP	Amyloid Precursor Protein
ATP	Adenosine Triphosphate
Aβ	beta-Amyloid
BSA	Bovine Serum Albumin
CAT	Catalase
C	Cytosine
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Egtazic Acid
FMRP	Fragile X Mental Retardation Protein
FXS	Fragile X Syndrome
FXTAS	Fragile X Tremor/Ataxia Syndrome
G	Guanine
GABA	γ-Aminobutyric Acid
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GTP	Guanosine Triphosphate
HBSS	Hank's Buffered Balance Solution
HIF	Hypoxia-Inducible Factor
KO	Knockout
L_N	Leak State
MD	Mitochondrial Dysfunction
mRNA	Messenger Ribonucleic Acid

NAD	Nicotinamide Adenine Dinucleotide
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOX	NADPH-Oxidase
OXPHOS	Oxidative Phosphorylation
P_{GM}	Oxidative Phosphorylation stimulated by Glutamate and Malate
P_{GMP}	Oxidative Phosphorylation stimulated by Glutamate, Malate, and Pyruvate
P_{GMPS}	Oxidative Phosphorylation stimulated by Glutamate, Malate, Pyruvate, and Succinate
P_{TM}	Oxidative Phosphorylation stimulated by Glutamate, Malate, Pyruvate, Succinate, Ascorbate and Tetramethyl-p-phenylenediamine
PCR	Polymerase Chain Reaction
PMSF	Phenylmethylsulfonyl Fluoride
RAGE	Receptor for Advance Glycation End-products
RIPA	Radioimmunoprecipitation Assay
RNS	Reactive Nitrogen Species
RORα	Retinoid-related Orphan Receptor-α
ROS	Reactive Oxygen Species
SEM	Standard Error of the Mean
SOD	Superoxide Dismutase
TBS-T	Tris Buffered Saline with Tween
TMPD	Tetramethyl-p-phenylenediamine
WT	Wild Type

Chapter 1: Introduction and Objectives

1.1 Fragile X Syndrome

1.1.1 Clinical presentation

Fragile X Syndrome (FXS) is the most common heritable cause of intellectual disability and a frequent form of Autism Spectrum Disorder. Individuals with this disorder suffer from cognitive and adaptive limitations such as attention deficit, anxiety, sleeping difficulties, motor disorders, and autistic disorders (reviewed in Raspa, Wheeler, & Riley, 2017). Furthermore, the phenotype is associated with a delay in language development, increased reactivity to sensory stimulation, and heightened propensity to seizures (reviewed in Till, 2010). Along with the cognitive limitations, the disorder has characteristic physical features such as a long and narrow face, large ears, a prominent forehead, and low muscle tone (Lachiewicz, Dawson, & Spiridigliozzi, 2000). FXS follows an X-linked dominant inheritance and due to this affects males at a higher rate than females. The prevalence rate of FXS is 1 in 4000 males and 1 in 8000 females (De Vries et al., 1997). Interestingly, the disorder presents differently across the sexes, with increased severity in males. Due to this, the disorder has been predominantly studied in males; however, investigations have shown that females affected by the disorder present with different cognitive and clinical characteristics (Rinehart, Cornish, & Tonge, 2010). Despite the prevalence and severity of this disorder there has yet to be an effective treatment or cure developed.

1.1.2 Brain abnormalities in Fragile X Syndrome

Beyond the clinical presentation of the disorder, studies have been completed to assist in understanding the pathology of FXS and how it affects the developing brain. Within the FXS brain, abnormal size, connectivity, and activity of multiple regions of the frontal cortex have

been identified (Hoeft et al, 2010). Furthermore, there is evidence indicating alterations in ventricles, cerebellar vermis, and brain glucose metabolism (Irwin et al., 2001). At the neuronal level, alterations include a higher density of dendritic spines, formation of long, thin and sinuous dendritic spines, increased synapse number and poor synaptic pruning (reviewed in Clarke & Barres, 2013). These deficits are thought to be the central target for the intellectual disability seen within the disorder. Interestingly, these inconsistencies and the clinical presentation of FXS are the result of a single gene mutation.

1.1.3 Genetics of Fragile X Syndrome

Genetically, FXS is characterised by the instability of a CGG-repeated tract at the 5' untranslated region of the *Fmr*1 transcript (reviewed in Raspa, Wheeler, & Riley, 2017). In healthy individuals, the normal range of CGG repeats is between 6 and 44, however, individuals with FXS have >200 repeats. The large amount of repetition leads to hypermethylation of the region during gestation, thus causing instability which disables transcription of FMRP (Fragile X mental retardation protein), a protein integral for normal brain development. FMRP is a mRNA translational regulator that is responsible for binding mRNA (Bechara, et al., 2009). In mice that do not express FMRP there have been several hundred mRNAs found with altered expression and localization. This altering of expression of many mRNAs has a large impact on neurodevelopment and ultimately results in the clinical presentation of FXS described above.

Since the *Fmr*1 transcript is found on the X chromosome, males with a full mutation have complete silencing and therefore no production of FMRP. However, in females the level of FMRP depends on the inactivation of the affected X chromosome. Due to the mosaicism of X-inactivation FXS can differentially affect females. In females with a full mutation, the disorder exists on a spectrum of severity inversely correlated with the levels of FMRP produced (Pretto et

al., 2014). The lack of FMRP is ultimately accountable for the pathology of FXS. However, much remains unknown regarding the cellular and molecular affects that occur due to the altered expression and localization of mRNAs in the FXS brain. One aspect of FXS pathology that has yet to be fully investigated is the presence of oxidative stress in the brain.

1.2 Oxidative Stress

1.2.1 Oxidative stress and the brain

Oxidative stress is defined as the imbalance between the production and elimination of free radicals (Liemburg-Apers, Willems, Koopman, & Grefte, 2015). Over abundance of free radicals leads to deleterious oxidative reactions causing DNA, protein, and tissue damage. Oxidative stress can occur throughout the body; however, the brain is particularly susceptible to its effects. The brain utilizes 20% of the total basal oxygen budget, meaning it is extremely metabolically active and relies heavily on oxygen for the production of ATP (Hyder, Rothman, & Bennet, 2013). Along with the reliance on oxygen for energy production, the brain requires appropriate levels of free radicals and reactive oxygen species for redox signalling. Redox signaling involves redox-sensitive molecules which respond to physiological levels of reactive oxygen species and can influence signaling proteins, cytoskeletal components, and transcription factors, making redox signalling an important aspect to brain functionality and development (reviewed in Bórquez et al., 2016). However, the levels of free radicals required for proper redox signalling and brain functioning are moderate or low amounts. If the amount of reactive species is excessive, they can be hazardous to surrounding cells and tissue. Due to the requirement of tight oxidative control within the brain it is clear that inadequate oxidative homeostasis would be detrimental to brain development and function. Furthermore, brain tissue has a very high composition of lipids. This makes the brain even more susceptible to the deleterious affects of

oxidative stress as a common, damaging reaction due to free radical over production is lipid peroxidation. Oxidative stress occurs due to improper control of free radicals and can cause serious damaging affects; therefore, it is important to understand the molecular and cellular processes which lead to this physiological burden.

1.2.2 Sources of oxidative stress

Major sources of free radical production within the brain include the mitochondria, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases, neurotoxicity, and insufficient elimination of free radicals due to low levels of antioxidants. The mitochondria are the primary source of ATP production by performing oxidative phosphorylation. Through this energy generating process they also create free radicals or reactive oxygen species (ROS), reactive nitrogen species (RNS), and carbon- and sulfur-centered radicals (Pero et al., 1990). These reactive species may potentially lead to oxidative stress if not appropriately handled by the antioxidant system, or if they overwhelm the capacity of the antioxidant system. Interestingly, mitochondrial ROS production may also stimulate the activation of NADPH-oxidase (NOX), another common source of free radical production in the brain (reviewed in Jiang, Zhang, & Dusting, 2011). NOX catalyzes the production of a superoxide free radical (O_2-) by transferring one electron to oxygen from NADPH, furthermore, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) are both coenzymes involved in maintaining the redox state of the cell (reviewed in Jiang, Zhang, & Dusting, 2011). There is evidence supporting crosstalk between NOX and mitochondria, however the exact processes remain unknown. Despite this, NOX still remains as a source of ROS production with increased activation being associated with oxidative stress. Increased NOX activation can occur for many reasons including up-regulation of expression, induction through stress signals, elevated intracellular Ca^{2+}, direct protein-protein interactions, or, as mentioned before, by mitochondrial

ROS production (reviewed in Jiang, Zhang, & Dusting, 2011). Although the mitochondria and NOX seem to affect each other, they both remain as important sources of free radical production worth investigating if interested in oxidative stress.

Another common source of cellular stress in the brain, and source of free radical production, is neurotoxicity. Neurotoxicity within the brain typically occurs due to the overproduction of glutamate, leading to tissue damage through lipid peroxidation (Herrera et al., 2007). Glial cells within the brain counteract the negative effects of glutamate toxicity by modulating the uptake of synaptically-released glutamate through glutamate uptake transporter activity (Mahmoud, Gharagozloo, Simard, & Grus, 2019). However, the capacity of these glial cells can be overwhelmed and/or their capacities may be deficient due to neurological stressors or disease.

The defence system in place to counteract all these causes of free radical production, is the antioxidant system. Antioxidants are a group of enzymes and low-molecular weight reductants which convert reactive species into harmless products. If there are ample antioxidants, they provide a buffer for oxidative stress by reducing the deleterious effects of the reactive species. However, improper, or inadequate levels of antioxidants can be overwhelmed by the production of reactive species and therefore lead to oxidative stress. Even during regular, healthy, oxidative processes, reactive species are produced. Therefore, simply a lack of antioxidants within a cell or tissue can be a cause of excessive reactive species leading to oxidative stress and damage. Overall, there are abundant sources of free radical production within the brain and while these processes occur naturally, in some cases they are associated with disease.

1.2.3 The contribution of oxidative stress to Fragile X Syndrome

Oxidative stress, and the resulting tissue damage, have been found to be a major contributor to many neurodevelopmental disorders. Specifically, in a review conducted by Valenti et al (2014) oxidative stress was found to be associated with Down Syndrome, Autism Spectrum Disorder, Rett Syndrome, as well as Fragile X- associated Tremor/Ataxia Syndrome (FXTAS) which, along with FXS, is characterized by CGG repeats in the *Fmr*1 gene. More recently, it has become evident that oxidative stress plays a role in the pathology of FXS. Firstly, oxidative stress is associated with anxiety, sleeping difficulties, and autism, all of which are associated with FXS (reviewed in Raspa, Wheeler, & Riley, 2017). Furthermore, the *Fmr*1 KO mouse model, a validated model for FXS which has complete silencing of the *Fmr*1 gene and no production of FMRP has been used to investigate oxidative stress. Specifically, within the brains of *Fmr*1 KO mice, evidence indicates higher levels of reactive oxygen species, NADPH-oxidase activation, lipid peroxidation, and protein oxidation (el Bekay, et al., 2007). This evidence suggests an imbalance between free radical production and/or the elimination of those free radicals. The cause of this imbalance, however, is difficult to locate. El Bekay et al (2007) found alterations with the antioxidant system of *Fmr*1 KO mice. Specifically, altered levels of components of the glutathione system which may contribute to the oxidative stress were seen. Elevated levels of glutamate, as mentioned before, can cause neurotoxicity and may also be a major effector of the oxidative stress in the FXS brain (Coyle & Puttfarcken, 1993). Interestingly, Wang et al (2016) found that neurotoxicity was triggered by an increased release of glutamate from *Fmr*1 KO astrocytes, suggesting an imbalance of glutamate in FXS. Furthermore, Shen et al (2019) uncovered mitochondria within *Fmr*1 neurons with dysfunctional fusion abilities, the first evidence of mitochondrial dysfunction within the FXS brain. Dysfunctional mitochondrial fusion results in mitochondrial fragmentation which has been found

to diminish the bioenergetic function of mitochondria (Chen et al. 2005). All these deficits may be associated with and contribute to the oxidative stress seen in FXS.

From the above evidence, oxidative stress plays a role within the pathology of FXS, however, much still remains unclear regarding the connection between the loss of FMRP and the cellular processes affected. Based on the literature, oxidative stress could be occurring within FXS due to an elevation of mitochondrial ROS production, the reduction of antioxidant enzymes, glutamate toxicity, and/or increased activity of NADPH-oxidase. Since much is still unknown about the cellular affects of losing FMRP it is important to consider all neural cell types and how they are affected. The cells that play the largest role in maintaining oxidative homeostasis within the brain are astrocytes.

1.3 Astrocytes

1.3.1 Role of astrocytes in neurodevelopment

Astrocytes are glial cells within the brain and have been found to be major participants in neurodevelopment of the central nervous system (reviewed in Clarke & Barres, 2013). They are the most abundant glial-cell type throughout the central nervous system (CNS) (Kettenmann & Ransom, 2005). Additionally, they provide the interface between the neural circuitry and the vasculature of the brain, with processes interacting with both synapses and blood vessels. Originally, astrocytes were thought to only provide passive, structural support within the brain, with neurons being the major cell involved with the development of neural circuitry. However, recently astrocytes have been found to play an integral role in synaptogenesis by facilitating synapse formation, inducing synapse maturation and controlling synapse pruning (reviewed in Clarke & Barres, 2013). Along with influencing these aspects of neural circuitry they have also been found to actively communicate with neurons through the secretion and uptake of neurotransmitters at the synapse. Specifically, studies have revealed that astrocyte-secreted

proteins induce and control synaptogenesis of both excitatory and inhibitory synapses (Kucukdereli et al., 2011; Elmariah, Oh, Hughes & Balice-Gordon, 2010). Furthermore, defects in astrocyte signalling and functionality have been found to be associated with many neurodevelopmental diseases, including FXS.

1.3.2 Role of astrocytes in oxidative homeostasis

Along with their importance throughout neurodevelopment, astrocytes also play an important role in protecting neurons at high levels of activity by acting as a buffer for oxidative stress (reviewed in Wilson, 1977). Oxidative stress occurs for many reasons, including dysregulation by antioxidants, increased NADPH-oxidase activity, neurotoxicity, and mitochondrial abnormalities. Astrocytes are capable of buffering oxidative stress by maintaining high intracellular concentrations of antioxidants. Specifically, astrocytes have the ability to synthesize and export glutathione to neurons (Chowdhury et al., 2018). Furthermore, astrocytes contain high amounts of small GTP-binding proteins which respond to changes in ROS and induce antioxidant genes for both superoxide dismutase (SOD) and NADPH oxidase (Messina, Di Zazzo, & Monmarchmont, 2017). Beyond these affects, antioxidant enzymes within astrocytes include superoxide dismutase (SOD), glutathione peroxidase (GPX), glutathione reductase (GR), and catalase (CAT), as well as low molecular weight reductants α-tocopherol (vitamin E), glutathione, and ascorbate (reduced vitamin C) (reviewed in Wilson, 1977).

Astrocytes also provide neuroprotection by acting to prevent glutamate toxicity within the CNS (Wilson, 1997; Coyle & Puttfarcken, 1993). Glutamate toxicity occurs when there is an overproduction of glutamate, which leads to oxidative damage and lipid peroxidation (Herrera, et al., 2007). Astrocytes counteract this through the uptake of synaptically-released glutamate which is mediated by glutamate uptake transporters predominantly found and expressed on and by astrocytes (Mahmoud, Gharagozloo, Simard, & Gris, 2019). Through this defence astrocytes

are capable of providing neuroprotection for oxidative stress throughout development of the brain, while also shaping the development of synapses.

1.3.3 Astrocytic deficits in Fragile X Syndrome

As mentioned earlier, defects in astrocyte signalling have been found to be associated with FXS. One study specifically investigated the impact of astrocytes on neural circuitry development within the context of FXS. By using co-cultures of neurons and astrocytes they discovered that when WT neurons were grown in co-culture with *Fmr*1 KO astrocytes they had deficits similar to *Fmr*1 KO neurons, with altered dendritic morphology and synaptic protein expression. Furthermore, they also discovered that when *Fmr*1 KO neurons were grown in co-culture with WT astrocytes they presented similar to healthy neurons without any of the aforementioned synaptic deficits (Cheng, Sourial, and Doering, 2012). This study exemplified the importance of astrocytes in neurodevelopment and how large of a role they play within the pathology of FXS. Another similar study performed by Hodges et al (2017) investigated the astrocyte's contribution to learning abnormalities within FXS. Using an astrocyte-specific *Fmr*1 KO mouse model they uncovered deficits in motor-skill learning and associated increases in dendritic spine density within the motor cortex. This study indicates further the effects that the lack of FMRP have on astrocytes and how that affects their ability to shape neurodevelopment.

Furthermore, within FXS, glutamate transporters have been found to be dysregulated in astrocytes and imbalances between glutamate and γ-aminobutyric acid (GABA) in astrocytes have been found to influence neuronal development (Higashimori, et al., 2016; Wang et al., 2016). These findings suggest a deficit in the *Fmr*1 KO astrocytes ability to mitigate glutamate toxicity which may be involved in the neuropathology of FXS. Collectively, these studies demonstrate that the loss of FMRP, as occurs in FXS, leads to impairment of astrocyte function in the developing brain. Due to the evidence of oxidative stress within the FXS brain, the

importance of astrocytes in maintaining oxidative homeostasis, and findings revealing astrocytic deficits within FXS, this thesis aims to examine astrocytic deficits in maintaining oxidative homeostasis within the FXS cortex with hopes of revealing new aspects of FXS pathology to assist future clinically-relevant studies in treatment development.

1.4 Hypothesis and Objectives

1.4.1 Hypothesis

In this study, I hypothesized that the absence of FMRP in cortical astrocytes leads to increased mitochondrial respiration and ROS production along with decreased expression of antioxidant enzymes and increased expression of NADPH-oxidase. Furthermore, the absence of FMRP in cortical tissue also leads to decreased expression of antioxidants and increased expression of NADPH-oxidase.

1.4.2 Objectives

In order to test these hypotheses, I aimed to address the following 4 objectives:

1) To characterize mitochondrial function and ROS production in astrocytes of the FXS mouse cortex. This was accomplished by using high-resolution respirometry of permeabilized cortical astrocytes from culture.
2) To examine the possible changes in NADPH-oxidase and antioxidant enzyme expression in the FXS cortex. To address this, western blots will be performed on cultured cortical astrocytes and cortical tissue.
3) To characterize mitochondrial function and ROS production in astrocytes of the FXS mouse cortex grown in different oxidative conditions. This will be assessed through maintenance of primary astrocyte cultures in both normoxia and hypoxia prior to performing high-resolution respirometry.

4) To characterize sex differences in mitochondrial function and ROS production in astrocytes of the FXS mouse cortex grown in different oxidative conditions. Both male and female derived primary astrocyte cultures will be used for high-resolution respirometry and analyzed.

These objectives are addressed in two separate chapters. Chapter 2 focuses on objectives 1 and 2, while chapter 3 focuses on objectives 3 and 4. These experiments are important in determining the effect the loss of FMRP has on the role of astrocytes in maintaining oxidative homeostasis.

Chapter 2: Aberrant production of astrocyte-mediated reactive species in Fragile X Syndrome

2.1 Introduction

During development, oxidative homeostasis is highly regulated within the nervous system and a tight balance of free radicals is important for many developmental processes (reviewed in Salim, 2017). However, deviations of either the production or elimination of free radicals results in oxidative stress that is associated with abnormal increases to reactive oxygen species (ROS) that can cause DNA damage, protein loss and lipid peroxidation (Cobley, Fiorello, & Bailey, 2018). Not surprisingly, several neurodevelopmental disorders have been associated with oxidative stress, including Autism Spectrum-related disorders, and changes in the degree of oxidative stress has been directly correlated with the severity of symptoms (Pangrazzi, Balasco, & Bozzi, 2020). In an animal model of Fragile X Syndrome, a genetic disorder highly comorbid with anxiety, hyperactivity and autism, higher levels of reactive oxygen species, an altered antioxidant system, and impaired mitochondrial ATP production have been found in various brain tissues (el Bekay, et al., 2007; D'Antoni, et al., 2019). The pathology that underlies abnormalities in redox regulation and oxidative stress in neurodevelopmental disorders like FXS remains unclear, but several reports have linked it to mitochondrial dysfunction within the CNS.

In a recent study on Fragile X Syndrome (FXS), impairment to mitochondrial fusion behaviour led to development deficits in newly formed hippocampal neurons (Shen et al., 2019). FXS is the most common inherited cause of intellectual disability and autism in children and is characterised by the instability of a CGG-repeated tract at the 5' end of the *Fmr*1 transcript that leads to hypermethylation and silencing of FMRP (Fragile X mental retardation protein). FMRP functions as a translational regulator for several hundred downstream proteins and is associated

with synaptic polyribosomes and RNA granules in neurons (Khayachi et al., 2018; Raspa, Wheeler, & Riley, 2017). Indeed, FMRP deficiency in the *Fmr1* KO mouse model resulted in a decrease in Hungtinin mRNA and protein levels within hippocampal neurons, which led to a decrease in mitochondrial fusion gene expression, impaired mitochondrial fusion, and reduced dendritic complexity and maturation (Shen et al., 2019). While this work demonstrated mitochondrial dysfunction and susceptibility of developing neurons to oxidative stress in the FXS brain, how these mechanisms affect other neural cell types that also rely on FMRP during brain development is unknown. In the CNS, astrocytes play a significant role in regulating oxidative homeostasis, with the ability to either exacerbate oxidative stress by serving as a source of ROS or modulate resistance by providing a prime source of antioxidants (Jou, 2008). Astrocytes normally express FMRP throughout development and a lack of FMRP has led to various impairments to astrocyte function, including changes to glutamate uptake and catabolism (Higashimori, et al., 2016). One primary goal of this study was to examine the consequence of FMRP deletion on mitochondrial function and ROS production in astrocytes in order to further elucidate their potential contribution to oxidative stress in FXS.

During periods of oxidative stress, the supply of astrocyte-mediated antioxidants is especially important for the protection of neurons susceptible to oxidative damage (Wilson, 1997). One particularly key role of astrocytes is the synthesis and exportation of glutathione to neurons (Wilson, 1997). Glutathione (GSH) acts to detoxify ROS within the brain, and deficiency of this tripeptide has been associated with mitochondrial damage and neurodegeneration (Jain et al., 1991; Bains & Shaw, 1997). Astrocytes not only produce high levels of GSH within the CNS but also supply neurons, which minimally express GSH, with the essential precursors for GSH synthesis (Dringen et al., 1999).

Furthermore, in response to high or low levels of ROS, astrocytes also induce the expression of superoxide dismutase (SOD) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, respectively (Wang et al, 2018A). SOD is responsible for the dismutation of superoxide within the cytoplasm and mitochondria, partitioning superoxide (O_2-) into oxygen (O_2) and hydrogen peroxide (H_2O_2), in order to reduce levels of ROS that can accumulate during oxidative phosphorylation (reviewed in Wang et al, 2018B). The expression of SOD in astrocytes appears particularly important to neuronal survival. In neuron astrocyte co-cultures, approximately half of the neurons die when cultured with SOD-mutant astrocytes as compared with wild-types, with the surviving neurons showing dramatic decreases of neuronal synaptic protein expression in the presence of glutamate (Kunze et al., 2013). Given that glutamate dysregulation has been associated with abnormal connectivity within the FXS brain, changes to SOD expression in astrocytes could act to exacerbate glutamate-mediated neuronal effects (Mahmoud et al, 2019). In contrast to SOD, NADPH oxidase in astrocytes produces intracellular ROS needed for redox signalling. Notably, overproduction of ROS via NADPH oxidase has been noted during frequent activations, and higher levels of activation of NADPH oxidase have been previously reported in FXS brains (Wang et al, 2018A; El Bekay et al., 2007).

Taken together, dysregulation of astrocyte-mediated antioxidant function in FXS may largely contribute to oxidative imbalance within the CNS and underscore many of downstream consequences to neuronal connectivity. In light of this, our study also examined several key components of the antioxidant systems within both primary astrocytes and the cortex of wild-type and *Fmr*1 KO mice during periods of peak astrocyte development and integration in order to promote our understanding of astrocyte-mediated pathophysiology in FXS.

2.2 Materials and Methods

2.2.1 Animals

Wildtype (WT) mice and *Fmr*1 KO mice (background FVB.129P2[B6]-Fmr1tm1Cgr) were housed and bred in the McMaster University Central Animal Facility. All animal-handling procedures and experiments followed the guidelines set by the Canadian Council on Animal Care and were approved by the McMaster Animal Research Ethics Board (Animal Utilization Protocol 17-04-11).

2.2.2 Tissue collection

Cortical tissue was isolated from the brains of WT and *Fmr*1 KO mice pups at postnatal day (P)1 to be used for protein measurements. Prior to cortical isolation pups were euthanized by decapitation and the brains were carefully removed and placed in a dish containing ice cold Hank's Balanced Buffer Solution (HBSS; Invitrogen, Carlsbad, CA, USA) supplemented with HEPES (Invitrogen). In this dish the cortices were isolated from the rest of the brain and immediately frozen in liquid nitrogen. Tissue was then stored at -80°C prior to protein measurements.

2.2.3 Cortical astrocyte culture and preparation

Primary astrocyte cultures were prepared from brains of three WT or three *Fmr*1 KO pups at P1-P3. Cortical tissue was isolated and mechanically homogenized with a scalpel blade and then chemically homogenized with 2.5% Trypsin (Invitrogen) in the presence of DNase I (Roche, Penzburg, Upper Bavaria, Germany). After thorough trituration and passage through a cell strainer (70 μm; Thermo Fisher Scientific, Waltham, Massachusetts, United States), the solution containing the cortical cells was centrifuged for 5 min at 1400 rpm. Supernatant was removed and the pellet was resuspended in minimum essential media (Invitrogen) supplemented with 6% glucose and 10% horse serum (Invitrogen). The cell suspension was plated in a T75

tissue culture flask and incubated at 37°C with 5% CO2 until 80-90% confluent (approximately 12-14 days) prior to use. Media changes of half volume occurred 24 hours after plating and then every two to three days.

At 80-90% cell confluence, the media was replaced with 0.05% trypsin-EDTA and glial media (minimum essential media supplemented with 6% glucose and 10% horse serum) and incubated for 5-10 minutes at 37° C. Following incubation, 5 mL of glial media was added to the flask and the solution was pipetted against the bottom of the flask to loosen cells. All of the liquid was removed and then centrifuged within a 15 mL falcon tube at 150-200 rpm for 5 minutes.

The cells used for real-time respiration studies were resuspended in 1 ml of respiration buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l−1 fatty acid-free bovine serum albumin; pH 7.1). After thorough trituration, 10 μL of the mixture was used to count the cells using a haemocytometer and a Zeiss Axioimager M2 (x10) while remainder of the cell suspension was maintained on ice. Once cell numbers were determined the solution was immediately used for high resolution respirometry. The cells that were collected for protein assays and western blots were resuspended in 1 mL of glial media. A 10 μL aliquot of the solution was applied to a haemocytometer and cells were counted using a Zeiss Axioimager M2 (x10). The remainder of the cell suspension was centrifuged at 1400 rpm for 5 minutes. The supernatant was removed and the tube containing the pellet was flash-frozen in liquid nitrogen prior to storage at -80° C. Notably, each sample (n) was obtained from primary cells that originated from individual litters (3 individuals per litter/sample).

2.2.4 Mitochondrial respiration and ROS production

Respiration and ROS production of primary cortical astrocytes were measured simultaneously using high-resolution respirometry (Oxygraph-2K, Oroboros Instruments, Innsbruck, Austria) in 2 ml of respiration buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l−1 fatty acid-free bovine serum albumin; pH 7.1) at a temperature of 37°C. After calibration, one million cells were added to each well and cells were permeabilized to allow substrates to cross the cell membrane unencumbered. To permeabilize the cells, 12 ul of a 1 mM digitonin solution was then added for a final concentration of 6 µM per well. The concentration of digitonin was previously optimized for this density of cells. After a 5-minute acclimation period, leak respiration was stimulated with the addition of 20 mM glutamate (Sigma-Aldrich, St. Louis, MO, USA) and 5 mM malate (Sigma-Aldrich) to each well and readings were recorded approximately 2 minutes after. After the addition of substrates, no further substrates were added until respiration rates and H_2O_2 emission rates stabilized (approximately 2 minutes). OXPHOS respiration in the presence of 20 mM glutamate and 5 mM malate was stimulated via multiple complexes of the electron transport system with progressive additions of 20 mM ADP (complex I; Sigma-Aldrich), 10 mM pyruvate (complex I; Sigma-Aldrich), 20 mM of succinate (complexes I + II; Sigma-Aldrich), and 2 mM of ascorbate (Sigma-Aldrich) and 0.5 mM of TMPD (complex IV; Sigma-Aldrich). It should be noted that after addition of ascorbate only respiration rates, not H_2O_2 emission rates, could be measured.

2.2.5 Microscopy

Primary cultured astrocytes labelled with Mitotracker Red CMXRos (Invitrogen) were imaged at 40x objective magnification using the Axio Observer Z1 inverted microscope (Carl Zeiss; Oberkochen, Germany), Axiocam 506 camera (Carl Zeiss), and Zen Blue (2.0) Software

(Carl Zeiss). Cells were plated onto coverslips coated with Poly-D-Lysine (Sigma-Aldrich; 1 mg/ml) and laminin (Invitrogen; 0.1 mg/ml) at a density of 8000 cells per coverslip. Cells were maintained in minimum essential media with 6% glucose (Sigma-Aldrich) and 10% horse serum (Invitrogen) on coverslips for 7 days *in vitro*. Forty-five minutes prior to imaging cells were incubated with 25 nM Mitotracker Red CMXRos (Invitrogen) in a phosphate buffered saline (Gibco; Gaithersburg, MD, US) at 37°C. Plates were then transferred to the recording chamber of the Axio Observer Z1 inverted microscope (Carl Zeiss) at 5% CO_2 and 37°C and imaged.

2.2.6 Enzyme Activities

Citrate synthase and cytochrome c oxidase activities were measured using a protocol adapted from Dawson et al. (2016). The maximal activities were assayed at 37°C for cultured primary astrocytes. Samples were homogenized in 10 volumes of ice-cold homogenizing buffer A [100 mmol l^{-1} KH2PO4 buffer, pH 7.2, containing 1 mmol l^{-1} EGTA, 1 mmol l^{-1} EDTA and 1 mmol l^{-1} phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were then centrifuged at 1000 g at 4°C and the supernatant collected for use in enzyme assays. Measured activities were assayed in triplicate. Enzyme activity was determined as the difference between the rate measured using all assay components and the background reaction rate. Measurements were carried out in 100 mmol l^{-1} KH2PO4 (pH 7.2) under the following assay condition. For cytochrome c oxidase, otherwise known as complex IV of the electron transport system: [CIV; ε=28.5 (mmol l^{-1}) $^{-1}$ cm^{-1} at 550 nm]: 0.2 mmol l^{-1} reduced cytochrome c (Calzyme Laboratories). For citrate synthase of the tricarboxylic acid cycle: [CS; ε=14.15 (mmol l^{-1})$^{-1}$ cm^{-1} at 412 nm]: 0.5 mmol l^{-1} oxaloacetate, 0.15 mmol l^{-1} acetyl-coA, 0.15 mmol l^{-1} 5,5′- dithiobis-2-nitrobenzoic acid. Enzyme activities were expressed in units of micromole substrate per cell per minute, with cell numbers determined using a haemocytometer and a Zeiss Axioimager M2 (x10; Carl Zeiss). Preliminary experiments determined that all substrate concentrations were saturating. Assays

were measured using an xMark Microplate Spectrophotometer (Biorad, Mississauga, ON, CAN). Data were analyzed using the accompanying Microplate Manager Software and GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Biochemicals were from Sigma-Aldrich unless otherwise stated.

2.2.7 Protein measurements

Protein assays were performed on both culture and cortical tissue samples in order to normalize of protein content used in western blotting. RIPA buffer was made by adding one tablet of protease inhibitor (Roche) and one tablet of phosphatase inhibitor (Roche) to 10 mL of the stock solution, which was kept on ice. Once defrosted, the samples were centrifuged at 10,000 rpm for 3 minutes at 4° C. Supernatant was removed and the pellets were mechanically homogenized in 20 μL of RIPA buffer with a Teflon pestle for several minutes. The homogenate was chilled on ice for 15 minutes, prior to centrifugation at 10,000 rpm for 10 minutes at 4° C. The supernatant was removed and transferred to a new tube and stored on ice until use in the DC protein assay.

For accurate measures of protein within the samples, we prepared a standard curve consisting of 4 serial dilutions of bovine serum albumin (BSA; Sigma-Aldrich, cat. no. A-7906) including: 0.2 mg/mL, 0.5 mg/mL, 0.8 mg/mL, and 1.1 mg/mL from a stock of 20 mg of BSA in 10 mL of water. The samples were centrifuged at 1000 x g for 15 minutes at 4° C. Sample aliquots were diluted 10x in RIPA buffer and 5 μL of each was added to a 25 μL mixture of Reagent A' and Reagent S to each well as per manufacturer's instructions (Bio-Rad, Hercules, CA, USA). 200 μL of Reagent B (Bio-Rad) was then added to all wells and the plate was gently shaken for 15 minutes prior to spectrophotometer readings (xMark Microplate Absorbance Spectrophotometer, Bio-Rad) taken at 750 nm. Samples were flash frozen in liquid nitrogen and stored at -80° C until further use.

After the amount of protein in each sample was determined, 30 µg of protein from each sample was combined with sample buffer: 2x Laemmli Buffer (Bio-Rad) and 5% 2-Mercaptoethanol (Sigma-Aldrich). Samples were heated for 5 minutes at 95°C, centrifuged briefly, loaded onto a gradient 4-15% precast polyacrylamide stain-free gel (Bio-Rad). Electrophoretic separation of proteins was done at 125V for 1 hour in a solution of 1X Tris-Glycine SDS running buffer (Bio-Rad). After electrophoresis, the gels were activated with UV light (302nm) for 1 minute and then transferred onto polyvinyl difluoride membranes (Bio-Rad) using the Trans-Blot Turbo Transfer System (Bio-Rad). The membranes were placed in a 1X tris buffered saline and tween-20 solution (TBS-T) and then imaged for total protein using a ChemiDoc Imaging System (Bio-Rad). Membranes were then incubated in a 5% non-fat milk solution in TBS-T for 1 hour at room temperature. Following this, membranes were rinsed with TBS-T and then incubated overnight at 4°C with TBS-T containing one of the following primary antibodies: anti-superoxide dismutase 1 (anti-SOD-1; rabbit; 1:5000; Abcam; ab13498), anti-SOD2/MnSOD (rabbit; 1:5000; Abcam; ab13533), anti-glutathione peroxidase 1 (anti-GPx; rabbit; 1:1000; Abcam; ab22604), anti-glutathione reductase (anti-GR; rabbit; 1:2000; Abcam; ab16801), anti-catalase (anti-CAT; rabbit; 1:2000; Abcam; ab16731), anti-nicotinamide adenine dinucleotide phosphate hydrogen (anti-NADPH) oxidase 2 (NOX2; rabbit; 1:5000; Abcam; ab129068), and anti-NADPH oxidase 4 (NOX4; rabbit; 1:500; Novus Biologicals; NB110-58851SS). Membranes were washed for 3 x10 minutes in TBS-T at room temperature, then incubated for 1 hr in horseradish peroxidase conjugated antibody (anti-rabbit, 1:2500, catalogue #: NA934-1ML; GE Healthcare Life Sciences, Mississauga, ON, CA). Membranes were washed for 3 x 10 minutes in TBS-T, then developed for 5 minutes using enhanced chemiluminesence (Bio-Rad) and imaged using a ChemiDoc Imaging system (BioRad). Analysis of relative densitometry was done using Image Lab Software 5.2 (Bio-Rad) and bands of interest were

normalized to total protein and a cross gel control for multiple gels. Normalized values were expressed as a relative percentage of WT densitometry values.

2.2.8 Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 6, with an α level set to 0.05. Data are presented as mean ± SEM. N represents the number of independent culture preparations or the number of mice tissue was collected from per condition. For individual comparisons, two-tailed unpaired t tests were performed and for multiple comparisons, ordinary two-way ANOVAs were performed.

2.3 Results

2.3.1 Mitochondrial respiration and ROS production in WT and Fmr1-KO cortical astrocytes

High resolution respirometry was performed on permeabilized cortical primary astrocytes in order to determine respiratory capacities across multiple mitochondrial electron transport complexes during both oxidative phosphorylation (OXPHOS) and leak state respiration. Respiration capacity of permeabilized cortical primary astrocytes in the *Fmr1* KO mice (n=12) was similar to that of WT mice (n=12) when OXPHOS (*green section*, Fig. 1A) was stimulated via ADP-stimulation and subsequent addition of glutamate, malate (Complex I: P_{GM}), pyruvate (Maximal Complex I: P_{GMP}), succinate (Complex II: P_{GMPS}), ascorbate, and TMPD (Complex IV: P_{Tm}) (Fig. 1C-D). These substrates, in conjunction with each other, stimulate various respiration states associated with mitochondrial electron transport complexes (as indicated above) and no differences in oxygen consumption were observed between WT and *Fmr1* KO cortical astrocytes during stimulation of CI - CII (P=0.3950; Fig. 1C) or CIV (P=0.1358; Fig. 1D). However, leak state respiration present in the absence of ADP (L_N; *red section*, Fig. 1A)

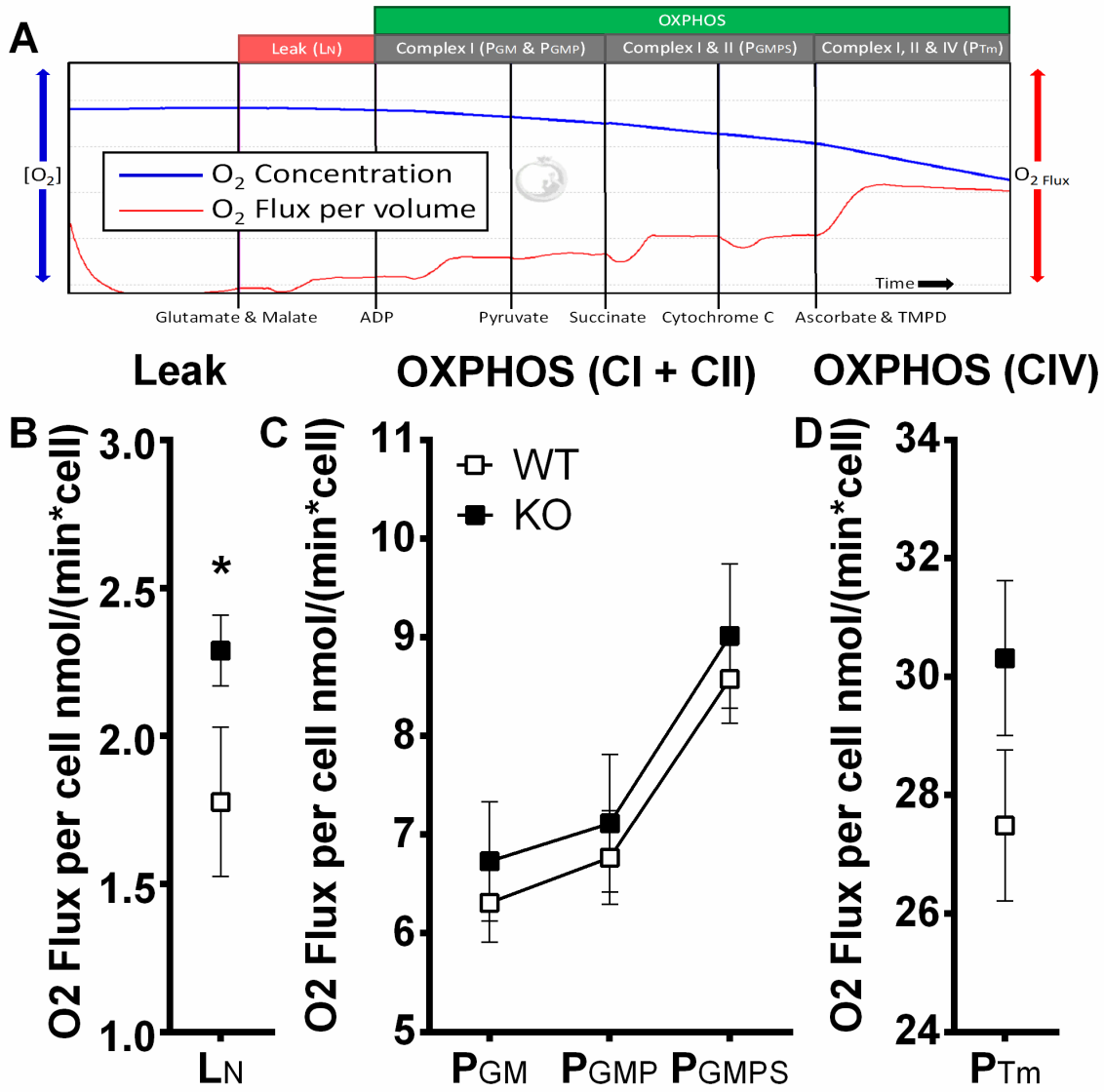

Figure 1. (*A*) Typical high resolution respirometry trace of WT oxygen consumption in permeabilized cultured astrocytes. Respiration rates of WT (n=13), and *Fmr*1 KO (n=14) permeabilized primary cortical astrocytes were stimulated via sequential application of exogenous substrates: addition of glutamate and malate for leak respiration (L_N); then ADP (P_{GM}) for oxidative phosphorylation via complex I; pyruvate (PGMP) for maximal stimulation of complex I; succinate (P_{GMPS}) for stimulation of complex II; and finally, ascorbate and TMPD (P_{Tm}) for stimulation of complex IV. *Fmr*1 KO astrocytes demonstrated significantly higher L_N values than WT (*B*), but no difference in oxidative phosphorylation capacity was measured (*C and D*). * Significant effect, P<0.05. Error bars, SEM.

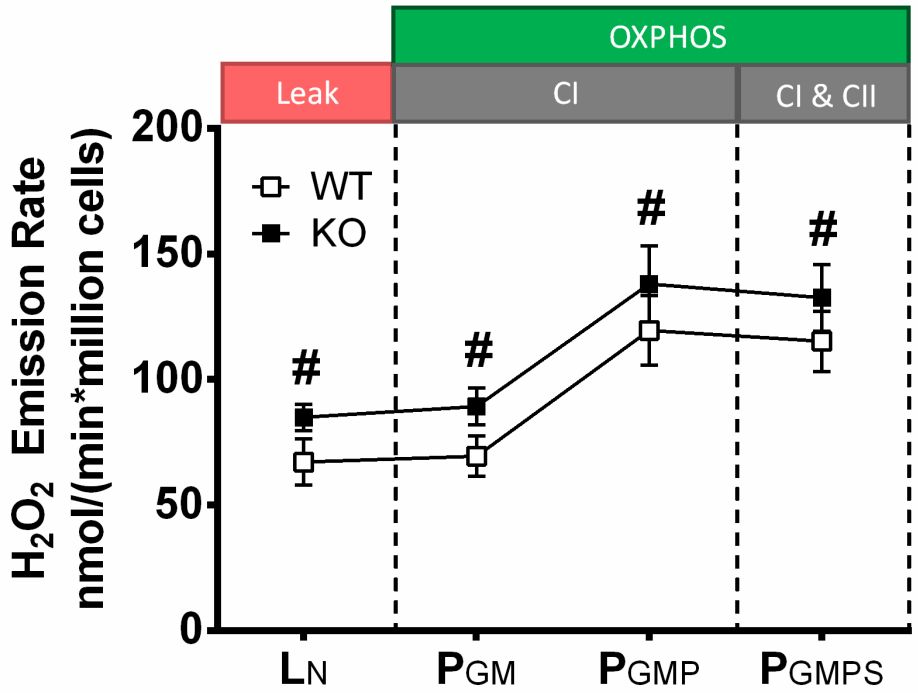

Figure 2. H_2O_2 emission rates of WT (n=9) permeabilized primary cortical astrocytes were significantly lower than that of *Fmr*1 KO astrocytes (n=11). Respiration states were stimulated as previously stated: leak respiration (L_N) via glutamate and malate; oxidative phosphorylation of complex I with glutamate, malate, and ADP (P_{GM}); maximal stimulation of complex I with glutamate, malate, ADP, and pyruvate (P_{GMP}); and stimulation of complex II with glutamate, malate, ADP, pyruvate, and succinate (P_{GMPS}) #- Significant main effect ($P<0.05$). Error bars, SEM.

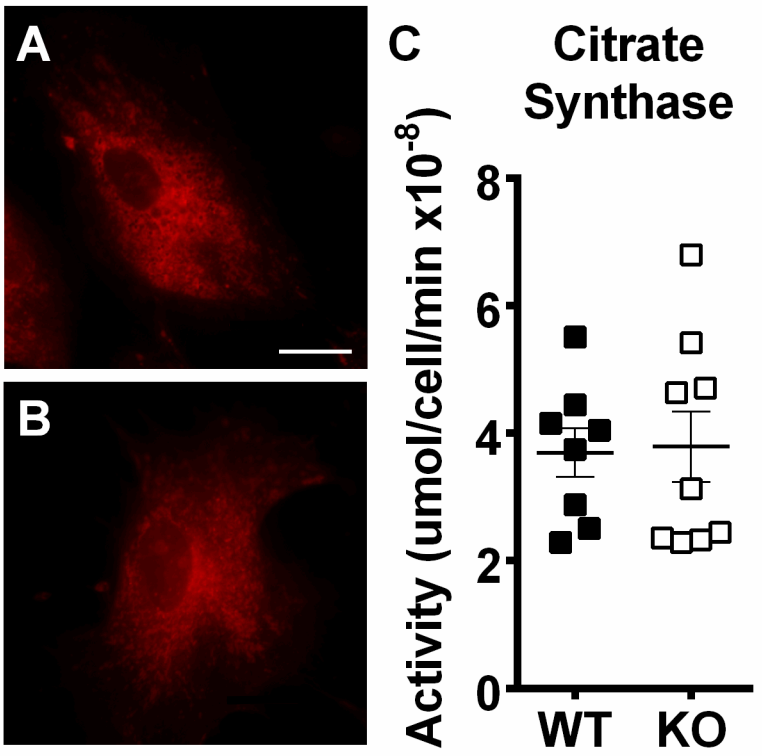

Figure 3. Representative images of WT (*A*) and *Fmr*1 KO (*B*) cortical cultured astrocytes stained with Mitotracker Red CMXRos. Scale bar, 20μm. (*C*) Activity of citrate synthase in WT (n=8) and Fmr1 KO (n=9) primary astrocytes. Astrocytes showed no significant genotypic difference in citrate synthase activity. Error bars, SEM.

was found to be significantly elevated in *Fmr1* KO cortical astrocytes compared to WT astrocytes (pairwise comparison, $P<0.05$; Fig. 1B). This state was mediated with the addition of glutamate and malate in the absence of ADP, disabling oxidative phosphorylation within the cells.

During leak state respiration (*red section*), the ROS production of permeabilized cortical astrocytes was significantly elevated in *Fmr1* KO (n=9) mice compared to WT (n=11) mice (Fig. 2). ROS production associated with leak state respiration and ADP-stimulated OXPHOS (*green section*) respiration with the subsequent addition of glutamate, malate (Complex I: P_{GM}), pyruvate (Maximal Complex I: P_{GMP}), and succinate (Complex II: P_{GMPS}) was also elevated in *Fmr1* KO cortical astrocytes compared to WT. Not surprisingly, ROS levels were significantly altered by different respiration states (main effect *respiration state*, $P < 0.0001$) and an overall significant difference between genotypes were observed across all respiration states and complex activation in two-way ANOVA (main effect of *genotype*, $P=0.0227$).

To ensure the differences we measured in respiration and ROS production were due to differential activity and not differential mitochondrial content across genotypes we performed a citrate synthase assay. Citrate synthase (CS) activity is a commonly used and validated biomarker for mitochondrial density (Vigelsø, Andersen, and Dela, 2014). Through this assay we determined that *Fmr*1 KO astrocytes had similar citrate synthase activity compared to WT astrocytes ($P=0.8923$; Fig. 3C). Furthermore, we completed a cytochrome c oxidase (complex IV) assay to support our respiration results. Through this assay we uncovered no genotypic differences in cytochrome c oxidase activity across *Fmr*1 KO (n=9) and WT (n=8) astrocytes ($P=0.4474$).

2.3.2 NADPH-Oxidase 2 and 4

Another common source of ROS production within cells is increased expression and activation of NADPH-oxidase (NOX). Therefore, we investigated the expression of two primary NADPH-oxidase isoforms (NOX2 and 4) in both cortical astrocyte and tissue preparations. We found that the expression of NOX2 was elevated in *Fmr*1 KO (n=8) cultured cortical astrocytes compared to WT (n=7) astrocytes (130.8 ± 8.47% of WT; $P<0.05$; Fig. 4A). Despite this elevation in astrocytes, the expression of NOX2 in cortical tissue was similar between WT (n=8) and *Fmr*1 KO (n=8) samples (99.12 ± 4.25% of WT; $P=0.8937$; Fig. 4B). This enzyme was highly expressed in both WT and *Fmr*1 KO cultured cortical astrocyte and cortical tissue samples. Furthermore, we found that the expression levels for NOX4 were similar in cultured cortical astrocytes across *Fmr*1 KO (n=8) and WT (n=8) mice (113.0 ± 32.26% of WT; $P=0.7938$; Fig. 4C). However, there was reduced expression of NOX4 in *Fmr*1 KO (n=8) cortical tissue compared to WT (n=8) cortical tissue (67.79 ± 5.21% of WT; $P<0.05$; Fig. 4D). This isoform was also highly expressed in both cultured cortical astrocyte and cortical tissue samples. Overall, we found an elevation of NOX2 in *Fmr*1 KO cultured cortical astrocytes with no differences in expression within cortical tissue. Along with this we found no differences in expression of NOX4 in cortical astrocytes but reduced expression of NOX4 in *Fmr*1 KO cortical tissue.

2.3.3 Antioxidant Expression in Cortical Astrocytes and Tissue

We investigated the expression of various key antioxidant enzymes present within astrocytes within the central nervous system. Those focused on in particular within this study are highlighted in Figure 5A. Enzyme expression of each was evaluated in both cortical tissue and primary cortical astrocytes isolated from *Fmr1* KO and WT mice (Fig. 5-7).

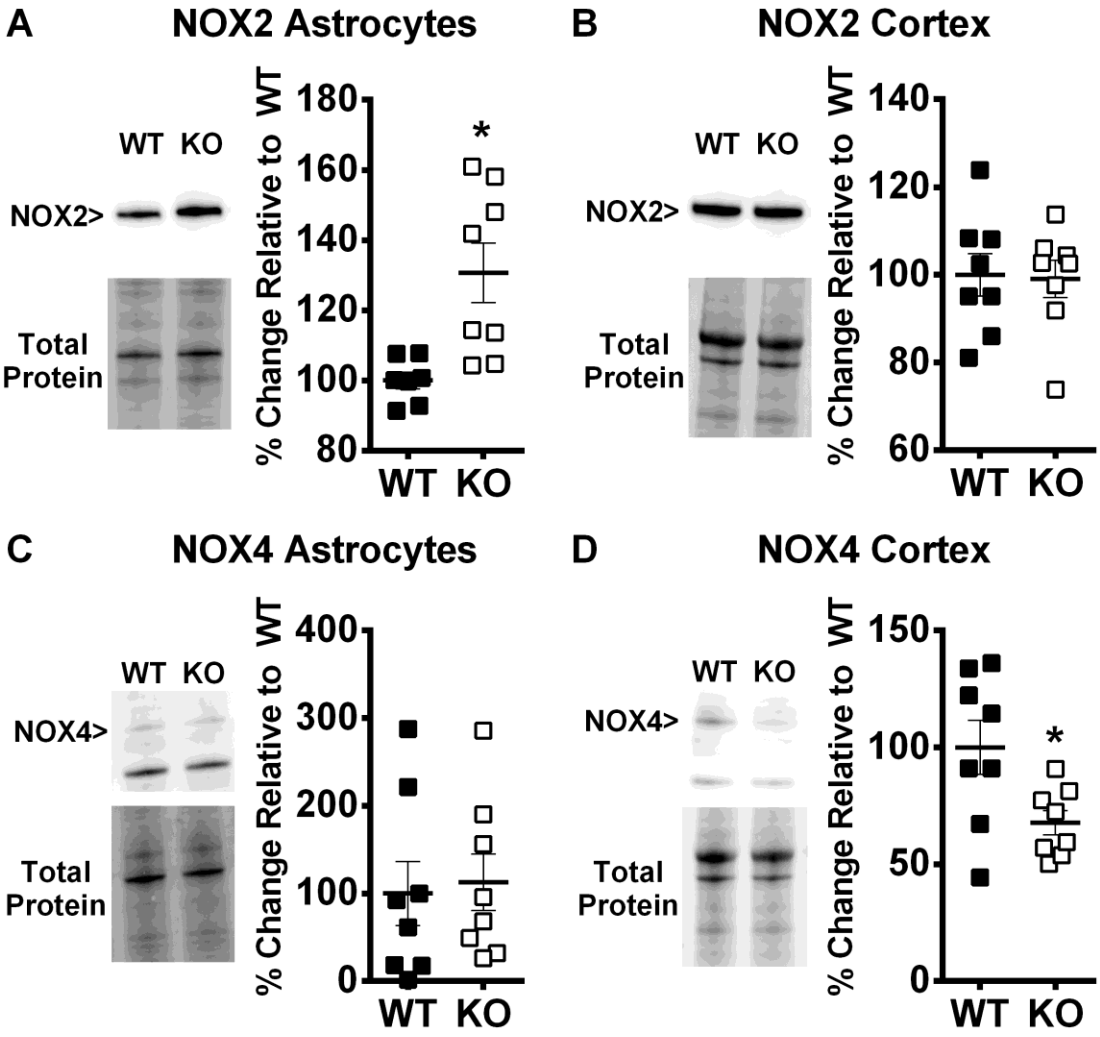

Figure 4. (*A*) Protein expression of NADPH-oxidase (NOX) 2 (~65 kDa) is elevated in *Fmr*1 KO (n=8) primary cortical astrocytes compared to WT (n=8). (*B*) There were no genotypic differences between WT (white) and *Fmr*1 KO (black) NOX2 expression observed in cortical tissue. (*C*) Protein expression of NOX4 (~70 kDa) in primary cortical astrocytes is similar across *Fmr*1 KO (n=8) and WT (n=8) mice. (*D*) Protein expression of NOX4 is decreased in *Fmr*1 KO (n=8) cortical tissue isolated from P1 mice comapred to WT cortical tissue. *- Significant effect ($P<0.05$). Error bars, SEM.

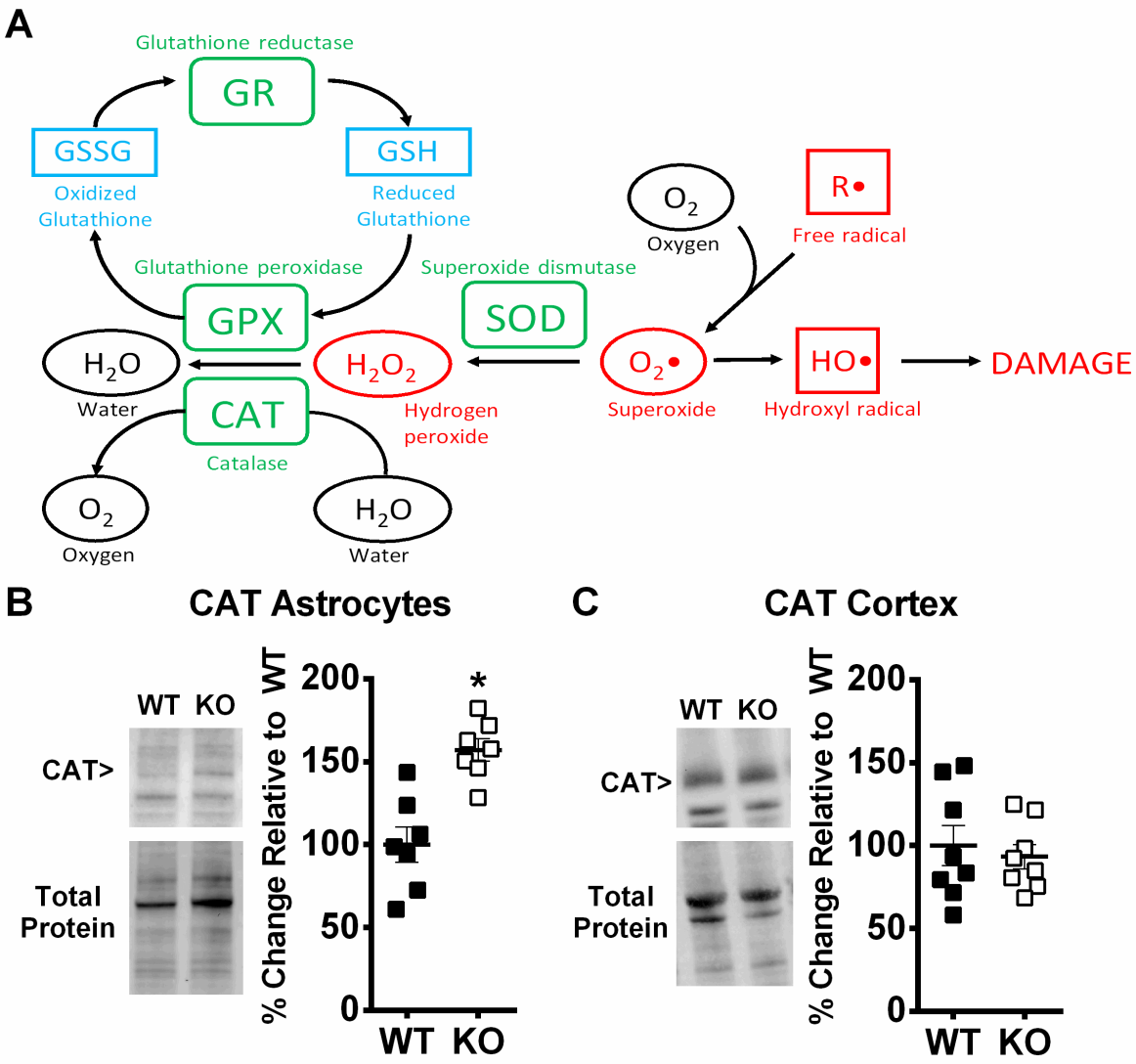

Figure 5. (*A*) Schematic of reactive oxygen species production and key antioxidant processes present in astrocytes to prevent oxidative damage. Protein expression of catalase (CAT; ~58 kDa) in primary astrocytes (*B;* n=7/group) was elevated in *Fmr*1 KO (black) mice compared to WT (white). P1 cortical tissue (*C;* n=8/group) showed no significant genotypic differences in antioxidant levels. *- Significant effect ($P<0.05$). Error bars, SEM.

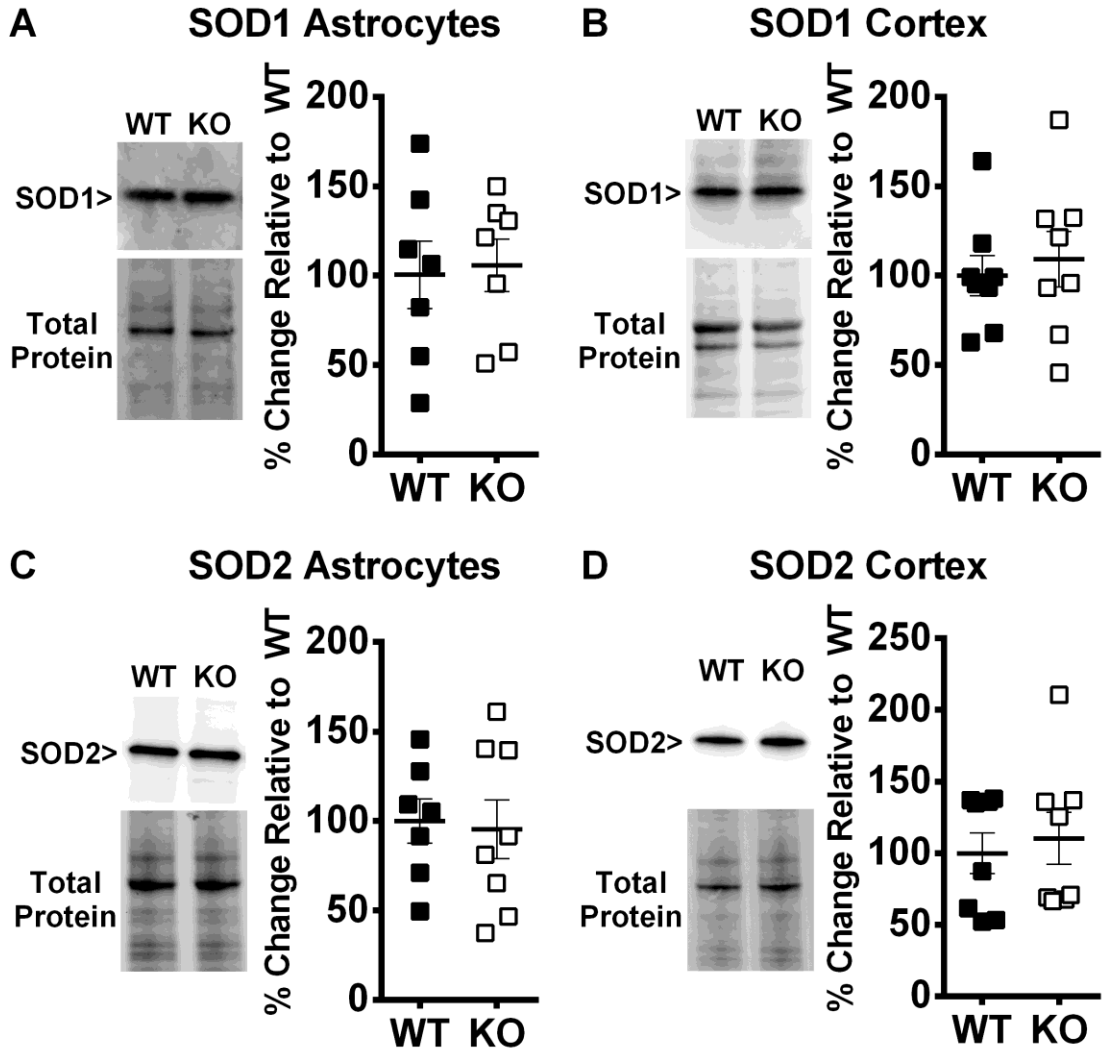

Figure 6. Protein expression of superoxide dismutase antioxidant enzymes in cultured primary astrocytes (n=7/group) and cortex (n=8/group) isolated from P1 WT and *Fmr*1 KO mice. No differences of superoxide dismutase 1 (SOD1;~19 kDa) or superoxide dismutase 2 (SOD2;~25 kDa) were observed between WT (white) and *Fmr*1 KO (black) primary astrocytes (*A and C*) or cortical tissue (*B and D*). Error bars, SEM.

Catalase (CAT)

CAT, an enzyme responsible for the conversion of hydrogen peroxide to water, is a vital component in reducing oxidative damage caused by ROS in astrocytes (Fig. 5A). We investigated the expression of CAT in primary cortical astrocytes and uncovered an elevation in *Fmr1* KO (n=7) mice compared to WT (n=7) mice (157.1 ± 6.66% of WT; $P<0.05$; Fig. 5B). There were no differences in CAT expression when *Fmr1* KO (n=8) and WT (n=8) cortical tissue was assessed ($P=0.8765$; Fig. 5C).

Superoxide Dismutase 1 and 2 (SOD)

Another important enzyme in the antioxidant system is superoxide dismutase (SOD). SOD is an enzyme responsible for the dismutation of superoxide into molecular oxygen and hydrogen peroxide. There are two different polymorphisms, Cu/Zn SOD1 is found within the cytosol and mitochondrial SOD2 is found within the mitochondria, both perform the same function in their respective parts of the cell (Fig. 5A). The expression levels of SOD1 in *Fmr*1 KO primary cortical astrocytes (n=7) was similar to expression levels in WT astrocytes (n=7) (105.8 ± 14.76% of WT; $P=0.8287$; Fig. 6A). This was also the case when comparing SOD1 in *Fmr*1 KO (n=8) and WT (n=8) cortical tissue ($P=0.6331$; Fig. 6B). Correspondingly, the expression levels of SOD2 in *Fmr*1 KO (n=7) primary cortical astrocytes were similar to that in WT (n=7) (95.4 ± 16.45% of WT; $P=0.8305$; Fig. 6C). There were also no differences in SOD2 expression between *Fmr*1 KO (n=8) and WT (n=8) cortical tissue observed ($P=0.4095$; Fig. 6D).

Glutathione Cycle Enzymes

One more integral component of the antioxidant system is the glutathione cycle. Glutathione peroxidase (GPx) works in sequence with glutathione reductase (GR) to catalyze the

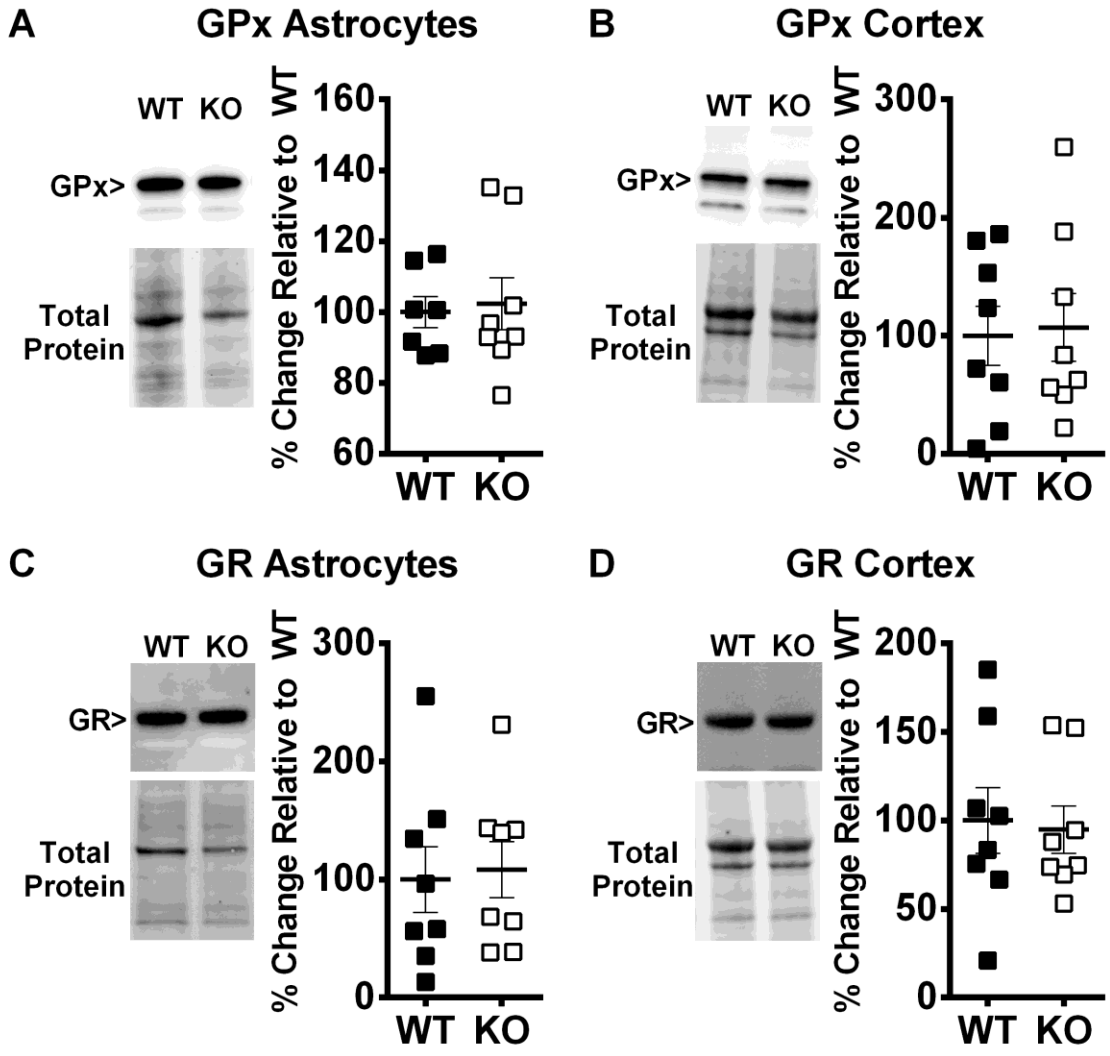

Figure 7. Protein expression of glutathione cycle antioxidant enzymes in cultured primary astrocytes (n=8/group) and cortex (n=8/group) isolated from P1 WT and Fmr1 KO mice. No differences of glutathione peroxidase (GPx;~22 kDa) or glutathione reductase (GR;~58 kDa) were observed between WT (white) and *Fmr*1 KO (black) primary astrocytes (*A and C*) or cortical tissue (*B and D*). Error bars, SEM.

reaction converting hydrogen peroxide to water, similar to the function of CAT (Fig. 5A). We found that the expression levels of GPx in both primary cortical astrocytes (*Fmr*1 KO, n=8; WT, n=7; *P*=0.7964) and cortical tissue (*Fmr*1 KO, n=8; WT, n=7; *P*=0.8587) were alike between genotypes (Fig. 7A-B). Similarly, the expression levels of GR in *Fmr*1 KO primary cortical astrocytes (n=8) were comparable to that of WT (n=8) (108.3 ± 23.79% of WT; *P*=0.8234; Fig. 7C). GR expression in *Fmr*1 KO cortical tissue (n=8) was also similar to cortical tissue isolated from WT mice (n=8) (*P*=0.8234; Fig. 7D). Notably, all antioxidant enzymes that we investigated were highly expressed in both WT and *Fmr*1 KO cultured cortical primary astrocytes as well as in the postnatal cortex; however, no genotypic differences were observed.

2.4 Discussion

In this study, we measured the mitochondrial respiration of primary cortical astrocytes isolated from WT and *Fmr1* KO mice to assess oxygen flux during induced leak state and oxidative phosphorylation (OXPHOS) across mitochondrial electron transport complexes. While OXPHOS capacity of the *Fmr1* KO astrocytes was overwhelmingly similar to that of WT astrocytes across successive stimulation of the protein complexes (I, II and IV); leak respiration of KO cells was significantly higher. Simultaneous readings of ROS production showed elevated levels in *Fmr1* KO astrocytes across each state of mitochondrial respiration compared to WT astrocytes, despite the comparable levels of oxygen consumption during OXPHOS. Interestingly, there was little genotypic difference in the overall expression of primary astrocyte enzymatic antioxidants that would offset the increase in ROS production in *Fmr1* KO astrocytes. There was, however, significant increases to the expression of NADPH oxidase 2, *NOX2,* in *Fmr1* KO astrocytes, which could augment ROS production observed in these cells. Collectively, our results demonstrate that ROS production is elevated within *Fmr1* KO cortical astrocytes

compared to their WT counterparts; yet, primary enzymatic antioxidant expression was not minimally elevated in response, suggesting that aberrant astrocyte activity contributes to oxidative imbalance within the FXS brain.

Oxidative stress pathology has been associated with a growing number of neurodevelopmental and neurodegenerative diseases. It is perhaps not surprising that previous studies have also demonstrated the presence of various oxidative stress indicators in the FXS brain. Enhanced levels of free radicals, as well as increased hydrogen peroxide production and lipid peroxidation activity were found in brain tissue of young adult *Fmr1* KO mice (el Bekay, et al., 2007; Diego-Otero et al., 2009). In conjunction with this, systemic treatment of α-Tocopherol, an antioxidant/radical scavenger, in *Fmr1* KO mice effectively reversed the appearance of oxidative stress markers in a dose-dependent manner (Diego-Otero et al., 2009). A more recent study by Shen and colleagues (2019), showed that knockdown of FMRP in neurons led to altered expression of several metabolic genes and impaired mitochondrial function. During development, synaptic transmission and dendritic spine development are highly dependent on mitochondrial morphology and bioenergetics (Flippo and Strack, 2017). In FXS, both synaptic function and dendritic spine maturation are impaired, an effect possibly due to reductions in mitochondrial membrane potential and fusion dynamics in *Fmr1* KO neurons (Shen et al., 2019). ROS overproduction leading to mitochondrial dysfunction has been found to activate the process of synaptic loss in drosophila, this provides a potential mechanism for the dendritic impairments seen in FXS (Beckhauser, Francis-Oliveira, & De Pasquale et al., 2016). Taken together, these studies show that the brain, and neurons in particular, are more susceptible to oxidative imbalances in Fragile X Syndrome and a further understanding of the underlying source of stress warrants further investigation.

Within the CNS, astrocytes are key homeostatic regulators responsible for neuronal protection as the microenvironments flux within the brain. In particular, they have key roles in regulating oxidative balance by providing a complete antioxidant response that clears free radicals produced by neurons and other cell types within the CNS (Stephen, Gupta-Agarwal, & Kittler, 2014). On the other hand, astrocytes also produce ROS and in some pathological conditions, ROS levels exceed the detoxifying capacity of the astrocytic antioxidant system and results in an excessive build-up of free radicals. Elevated levels of ROS are associated with reactive astrogliosis, a process of morphological and functional changes astrocytes undergo in response to acute stress or damage. While the definition of astrogliosis is still incomplete, astrocytes within this state for prolonged periods are known to sustain high levels of ROS production, secrete proinflammatory molecules and promote neurotoxicity (reviewed in Rizor et al., 2019). Markers used to characterize reactive astrogliosis, such as glial fibrillary associated protein (GFAP), and pro-inflammatory factors, including tumor necrosis factor 2, are both elevated across several brain regions in the *Fmr1* KO brain (Yuskaitis, Beurel and Jope, 2010; Pacey et al., 2015). In addition, *Fmr1* KO astrocytes grown in isolation express enhanced levels of tenascin C and cytokine interleukin-6, factors that have both been associated with the induction of inflammation in other neurological diseases (Krasovska and Doering, 2018). The significant elevations of ROS in *Fmr1* KO cortical astrocytes observed here indicate that astrocytes are persistently activated within the FXS brain, and likely contribute to the oxidative pathogenesis of this disease.

The source of free radicals within astrocytes is primarily due to ROS production by mitochondria or the NOX pathway. The mitochondria are the greatest source of ROS in astrocytes and is largely dependent on the function of the mitochondrial respiratory chain (MRC) during oxidative phosphorylation, particularly at complex I (Zhao, Jiang, Zhang, and Yu, 2019).

Interestingly, the MRC of astrocytes is markedly different than other CNS cell-types, such as neurons, that depend on OXPHOS for ATP production. In neurons, complex I is predominantly assembled into supercomplexes that support higher mitochondrial respiration with low ROS production (Bianchi et al., 2004). In contrast, the abundance of free, non-bound complex I is higher in astrocytes, and this directly correlates with substantially higher ROS production by comparison (Lopez-Fabuel et al., 2016). Whether MRC organization is altered in FXS is unknown, but hyperactivation of MRC complexes and compromised ATP production in the cerebral cortex of *Fmr1* KO mice has been reported (D'Antoni et al., 2019). The differences to ROS production in *Fmr1* KO astrocytes we observed don't appear to be due to any differences in mitochondrial matrix mass suggested by citrate synthase activity, or to any changes to organization as mitochondria distribution was analogous in both genotypes. The fact that similar levels of induced OXPHOS rates in *Fmr1* KO and WT permeabilized astrocytes resulted in significantly higher levels of ROS in *Fmr1* KO cells, suggests that either the MRC produces excess superoxides in FXS, the ROS source affected is not related to OXPHOS, or the antioxidant response is impaired.

In contrast to OXPHOS, oxygen consumption associated with leak state respiration in the absence of adenylates was significantly greater in *Fmr1* KO astrocytes when compared to WT. Several factors influence leak (state 4) respiration, including proton leak, proton slip, cation cycling and electron leak, and affect the protonmotive force (Δp) across the mitochondrial inner membrane (Gnaiger, 2014). The shift in protonmotive force within *Fmr1* KO astrocytes may be associated with increased ROS production, since increased leak state respiration can be driven by increasing ROS levels (Brookes, 2005). Since increases of basal respiration in state 4 are often indicative of increased proton leak, we also evaluated mitochondrial membrane integrity via cytochrome oxidase activity and found that changes to L_N respiration in *Fmr1* KO astrocytes

were not due to mitochondrial membrane damage in our preparation. Notably, in some disease conditions associated with elevated superoxide levels in the mitochondrial matrix, the cell increases proton conduction through upcoupling proteins in order to limit ROS overproduction (D'Ambrosio et al., 1993; Korge et al., 2003). While not studied in astrocytes, increased leakage across the inner membrane of neuronal mitochondria have been identified in the forebrain of *Fmr1* KO mice (Griffiths et al., 2018). Of note, skin fibroblasts isolated from patients with fragile X-associated tremor/ataxia syndrome (FXTAS), that results from a premutation expansion to the *Fmr1* gene, showed remarkably similar increases to state 4 respiration (~2X) seen here in comparison to healthy controls (Ross-Inta et al., 2010). In all, the changes to basal respiration noted in this study are in line with observations made in other neural cells and related disease models.

NADPH-oxidized pathway is another important source of ROS production within astrocytes. This family of enzymes contains 7 members, with NOX2 and 4 as the most abundant in the brain (Sorce et al., 2017). Within the cell, NOX enzymes are expressed at the cell surface as well as intracellular organelles, including the endoplasmic reticulum, endosomes and vesicles. Both NOX2 and 4 are expressed by astrocytes to varying degrees at rest but increase expression and activity in response to stress. For instance, astrocytic NOX2 is significantly upregulated by increasing amyloid-β in Alzheimer's disease and induces astrogliosis (Chay et al., 2017). Other stresses, such as lipopolysaccharide exposure or hypo-osmotic swelling, also leads to substantial increases of ROS production via NOX activation in astrocytes (Reinehr et al., 2007). Previous studies have reported greater NADPH-oxidase activity within the FXS brain (el Bekay, et al., 2007). We found NOX2 expression significantly upregulated in *Fmr1* KO astrocytes but not in

the cortex overall. Since NOX2 is highly expressed by a multitude of cell-types within the CNS, changes within astrocytes alone may not have been detectable at the tissue level.

Surprisingly, NOX4 expression appeared to be reduced in *Fmr1* cortical tissue. Like NOX2, NOX4 is also expressed on several cell-types within the CNS but to a much lower extent in astrocytes (Gray et al., 2015). Unlike NOX2, this enzyme is largely expressed within the mitochondria and contains an ATP-binding motif, which when bound, negatively regulates NOX4 activity (Shanmugasundaram et al., 2017). It is plausible that MRC hyperactivity in the FXS cortex could work to reduce NOX4 within mitochondria but presumably not if ATP production is impaired. While these changes indicate dysregulation of this enzyme family in FXS, future studies that systematically target NOX2 and 4 in *Fmr1* KO astrocytes *in vivo* will help to parse out their relative roles in ROS production.

In the CNS, astrocytes have a robust antioxidant system that works to offset increases to free radical production and aids in neuroprotection during excitatory transmission. Glutamate is the primary excitatory neurotransmitter within the CNS, but if high amounts remain within the synaptic cleft, overactivation of glutamate receptors leads to calcium overload, generation of ROS and neurotoxicity (Reynolds and Hastings, 1995). In defence, astrocytes actively transport glutamate out of the cleft via the glutamate transporters GLAST, GLT-1 and EAAC1. Expression of GLT-1 in particular is significantly reduced in FMRP deficient astrocytes, which leads to impaired glutamate uptake and enhanced neuronal excitability within the somatosensory cortex, a hallmark of FXS (Higashimori et al., 2013; 2016). Astrocytes also protect neurons by neutralizing the resultant hydrogen peroxide within the synaptic zone with antioxidant enzymes. Regulating the expression of many antioxidant enzymes is nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that only translocates to the nucleus upon activation by

ROS (Cuadrado et al., 2019). Translocation of Nrf2 is impaired within the *Fmr1* KO brain; however, pharmacological activation of Nrf2 restores the ratio of nuclear to cytosolic levels of this protein in the *Fmr1* KO brain that is correlated to behavioural improvement (Biekofsky et al., 2017). Thus, impairment of the antioxidant system appeared to be likely in *Fmr1* KO astrocytes.

The antioxidant glutathione (GSH) pathway is heavily regulated by Nrf2, particularly during the de novo synthesis of glutathione (Harvey et al., 2009). Glutathione acts in conjunction with glutathione peroxidase (GPx) to catalyse the reduction of hydrogen peroxide and peroxide radicals. El Bekay et al (2007) found that expression and activity of glutathione peroxidase was significantly reduced in 4-month-old *Fmr*1 KO mice brains. In contrast, we found that neither the expression of glutathione peroxidase or glutathione reductase, the enzyme that reduces glutathione disulfide to form glutathione, were altered in *Fmr*1 KO astrocytes or cortical tissue in comparison to WT. It appears that dyregulation of GPx must only occur at later time points in development since differences between *Fmr*1 KO and WT brain tissue have not been found in earlier postnatal stages (our observations; El Bekay et al., 2007).

Other key antioxidant enzymes regulated by ROS levels and Nfr2 are catalase and superoxide dismutase (SOD). Interestingly, we did find that the expression of catalase was significantly elevated in *Fmr*1 KO astrocytes in comparison to WT cells. Catalase, also responsible for the reduction of hydrogen peroxide within the cell, is found at a relatively low abundance within subcellular cytoplasm and peroxisomes in the mammalian brain (Mavelli et al., 1982). Given the low expression of catalase within the brain, the increases within *Fmr*1 KO astrocytes may not be enough to overcome the ROS production rate. It should be noted that in our permeabilized cells, much of the cytoplasmic catalase would be washed away. However, the

fact that there were no differences between the genotypes in catalase expression within cortical tissue suggests the elevations in astrocytes were not enough to be detected within tissue.

The other family of enzymes, superoxide dismutase (SOD), occurs in two different polymorphisms: Cu/Zn and mitochondrial SOD (SOD1 and SOD2, respectively). These work to neutralize superoxides into hydrogen peroxide and O_2^-, which are then reduced by other enzymes, such as catalase. Both forms are expressed widely within the CNS, SOD1 being within the cellular cytosol and SOD2 in the mitochondria. FMRP is known to regulate the translation of SOD1 protein within the brain, and expression of SOD1 is significantly reduced in several brain regions in postnatal *Fmr1* KO mice compared to WT (Bechara et al., 2009). Since FMRP expression is quite high postnatally within the brain, it was surprising that we did not see any genetic differences in either form of SOD within the cortical tissue or astrocyte culture. This suggests that while FMRP appears to be an important regulator of SOD1 (40% reduction in *Fmr1* KO), other regulatory factors are able to compensate for the loss of FMRP expression in our FXS animal model.

In all, our observations support previous findings that oxidative imbalance plays a role in the pathology of FXS. Furthermore, astrocytes seem to be an integral component that contribute to the loss of oxidative homeostasis within the FXS cortex rather than prevent it.

Chapter 3: Mitochondrial bioenergetics of astrocytes in Fragile X Syndrome

3.1 Introduction

Fragile X Syndrome (FXS) is a common form of Autism Spectrum Disorder and the most prominent genetic cause of intellectual disability with no available treatment (Penagarikano, Mulle, & Warren, 2007). Individuals with FXS suffer from cognitive and adaptive limitations, such as attention deficit, anxiety, sleeping difficulties, motor disorders and autistic disorders (reviewed in Raspa, Wheeler, & Riley, 2017). It is characterized by a mutation on the *Fmr*1 transcript of the X chromosome resulting in large amounts (>200) of CGG repeats. This sequence of nucleic acids causes hypermethylation and ultimately silences the gene coding for FMRP (Fragile X mental retardation protein). FMRP is a translational regulator which controls the expression of many functionally relevant downstream proteins (Raspa, Wheeler, & Riley, 2017). One consequence of protein dysregulation in FXS may be oxidative stress within the brain. Currently, oxidative stress has been associated with many Autism Spectrum-related disorders and other related neurodevelopmental disorders but only a handful of studies have linked this genetic mutation to oxidative stress related pathology.

Studies using the *Fmr*1 knockout (KO) mouse model of FXS have found that brain tissue contains higher levels of reactive oxygen species, altered levels of antioxidants, and impaired mitochondrial ATP production in young adults (el Bekay, et al., 2007; D'Antoni, et al., 2019). Furthermore, Shen et al (2019) uncovered evidence of oxidative stress within *Fmr*1 KO neurons by revealing the presence of increased superoxide along with decreased mitochondrial fusion gene expression, impaired mitochondrial fusion, and reduced dendritic complexity and maturation. This evidence demonstrated the impact of mitochondrial dysfunction on neurons

within the FXS brain. However, how the loss of FRMP affects mitochondrial function or oxidative homeostasis of other neural cell types within FXS remains unclear.

Glial cells have a very significant role in maintaining oxidative homeostasis in the brain, specifically, astrocytes regulate oxidative homeostasis through mitochondrial ROS production and maintaining antioxidants within the central nervous system (CNS) (reviewed in Wilson, 1997). Furthermore, FMRP is widely expressed in astrocytes during neurodevelopment (reviewed in Clarke & Barres, 2013). Therefore, it is likely that mitochondrial function would be impaired in this cell type since mitochondrial dysfunction has been found in *Fmr*1 KO neurons (Shen et al., 2019). Previous investigations uncovered increased leak state respiration and ROS production in *Fmr*1 KO astrocytes, suggesting mitochondrial abnormalities (Chapter 2, Fig. 2). However, these investigations were performed on primary astrocyte cultures prepared in atmospheric oxygen levels.

Most in vitro studies of astrocytes have been performed under hyperoxic conditions, meaning they have been grown in very high amounts of oxygen compared to in vivo. The brain begins to develop in conditions of hypoxia, or low oxygen (1-8% O_2), which then transitions to normoxic conditions following birth (Ikonomidou & Kaindl, 2011). In humans, this switch directly corresponds with peaks in the rate of tissue growth and metabolic demand of the CNS (Kuzawa, et al., 2014). Along with their role in maintaining oxidative homeostasis, astrocytes contribute greatly to CNS metabolism (Hertz et al., 2007). Interestingly, compared to neurons, astrocytes sustain a lower rate of oxidative mitochondrial metabolism and instead rely on glycolysis for energy production (reviewed in Belanger et al., 2011). Furthermore, astrocytes produce significantly more ROS than neurons (reviewed in Chen et al., 2020). Therefore, understanding how different oxygen tensions impact the regulation of oxidative homeostasis by

astrocytes is vital. Traditionally culture protocols recommended atmospheric oxygen levels of 21%, which is much higher than the normal physiological environment (Pavlacky & Polak, 2020). Therefore, both normoxic (21% oxygen) and hypoxic (3% oxygen) growth conditions were used to study astrocyte mitochondrial metabolism within the context of FXS. We hypothesized that oxygen levels, and their impact on astrocyte metabolism, will differentially effect WT and *Fmr*1 KO astrocyte-mediated respiration and ROS production. Furthermore, we assume the hypoxic condition of 3% oxygen to be more physiologically relevant and more accurately portray the physiology of cortical astrocytes in the brain, whereas the normoxic condition portrays cortical astrocytes with excessive, potentially stress inducing, levels of oxygen.

FXS is an X-linked dominant disorder, thus it differentially affects males and females at a rate of approximately 1 in 4000 and 1 in 8000, respectively (reviewed in Warren & Sherman, 2001). Furthermore, FXS has been predominantly studied in males with females showing different cognitive and clinical profiles (reviewed in Rinehart, Cornish, & Tonge, 2010). Therefore, sex differences are a particularly important consideration within the context of FXS. Estrogen, a hormone found predominantly in females, has a neuroprotective role in the CNS and can be synthesized by astrocytes (Arnold & Beyer, 2009). Estrogen induces the expression of antioxidant proteins such as SOD and glutathione peroxidase (GPX), and decreases mitochondrial ROS production (Arnold & Beyer, 2009). Oxidative damage to mitochondrial DNA in males is also higher than in females, suggesting the important role of estrogen in mediating expression of antioxidants (Borrás et al., 2003). Furthermore, estradiol, a form of estrogen, has been shown to exert regulatory actions on astrocytes such as regulating morphology and function (Guo et al., 2012). Based on these essential functions of estrogen in females and the greater genetic impact of FXS on males, we hypothesized that astrocytes isolated

from males would display increased ROS production compared to their female counterparts and that male *Fmr*1 KO astrocytes would be impacted to the greatest extent. Overall, this study evaluated how sex and oxidative culture conditions impact the mitochondrial function of both WT and *Fmr*1 KO astrocytes.

3.2 Materials and Methods

3.2.1 Animals

Wildtype (WT) mice and mice that do not express FMRP (*Fmr*1 KO mice; background FVB.129P2[B6]-Fmr1tm1Cgr) were housed and bred in the McMaster University Central Animal Facility. All animal-handling procedures and experiments were approved by the McMaster Animal Research Ethics Board (Animal Utilization Protocol 17-04-11) and followed the guidelines set by the Canadian Council on Animal Care.

3.2.2 Sex Determination

Determination of pup sex prior to culturing was performed by visualization of the anogenital distance. The anogenital distance is greater in males compared to females. To improve accuracy all pups from a litter we assessed, the ones with the greatest distance were used for male culture preparations, whereas the pups with the smallest anogenital distance were used for female culture preparations. This sex determination was also confirmed by polymerase chain reaction (PCR).

3.2.3 DNA Extraction and PCR

To extract DNA from mouse tails, REDExtract-N-Amp tissue PCR kit protocol was utilized (Sigma-Aldrich, St. Louis, MO, USA). Extraction solution of 100 uL was combined with 25 uL of tissue preparation solution in a small centrifuge tube and mixed well. Mouse tail clippings of 0.5-1 cm pieces were placed into the solution and incubated at room temperature for

10 minutes. The samples were then further incubated at 95°C for 3 minutes. Neutralization solution B of 100 uL was added to the samples and the samples were stored at 4°C until used for PCR.

For PCR amplification, 2 uL of nuclease- free water (Invitrogen, catalogue #10977015) was added to PCR tubes. REDExtract-N-Amp PCR reaction mix of 10 uL was then added to the tubes, followed by 4 uL of the DNA extracted tissue solution. 2 uL of forward primer (SRYf 15-3940; Integrated DNA technologies (IDT), Coralville, Iowa, USA) was added to the mix, along with 2 uL of reverse prime (SRYr 15-3941; IDT). This master mix was then used to perform PCR amplification. The cycling parameters for PCR amplification include an initial denaturation at 94°C for 3 minutes for 1 cycle. Then, denaturation at 94°C for 0.5-1 minute, annealing at 50°C for 0.5-1 minute, and extension at 72°C for 1-2 minutes for 30-35 cycles. This is followed by a final extension at 72°C for 10 minutes for 1 cycle. The samples are stored at 4°C until used for gel electrophoresis.

3.2.4 Cortical Astrocyte Culture

Either three WT or three *Fmr*1 KO pups at postnatal day (P)1-P3 were used for primary cortical astrocyte cultures. Pups were euthanized by decapitation and the brains were removed and placed in a dish with ice cold Hank's Balanced Buffer Solution (HBSS; Invitrogen, Carlsbad, CA, USA) supplemented with HEPES (Invitrogen). After brain removal, cortical tissue was isolated from the rest of the brain. This tissue was then homogenized with a scalpel and placed in a solution containing 2.5% Trypsin (Invitrogen) and DNase I (Roche, Penzburg, Upper Bavaria, Germany). After thorough trituration of this mixture the solution was passed through a cell strainer and then centrifuged for 5 min at 1400 rpm. At this time, the supernatant was removed, and the pellet resuspended in glial media which is a combination of minimum essential

media (Invitrogen), 6% glucose, and 10% horse serum (Invitrogen). The cells were then plated in this solution on a T75 tissue culture flask and incubated at 37°C with 5% CO_2 and 21% O_2 for 24 hours. To promote growth, media changes were performed 24 hours after plating and every 2-3 days after that, in which half the volume was removed and replaced with fresh glial media. After the first 24 hours of incubation, cultures were either placed in an incubator with 21% O_2 or an incubator with 3% O_2, these different conditions represent normoxia and physiological hypoxia, respectively. Cultures in both these conditions were incubated at 37°C with 5% CO2 until 80-90% confluent (7-14 days) prior to use.

3.2.5 Cortical Astrocyte Collection

Once primary cortical astrocyte cultures reached 80-90% confluency, the culture flask was removed from the incubator in preparation for collection of the cells to be used for high resolution respirometry measurements. After firmly tapping the bottom of the culture flask and removing existing glial media (minimum essential media supplemented with 6% glucose and 10% horse serum), the flask was washed with 5 ml of 0.05% trypsin-EDTA previously warmed to 37°C. The initial 5 ml of 0.05% trypsin-EDTA was removed and another 5 ml was added before incubating the flask at 37°C for 10 minutes. At the end of the incubation period, 5 ml of glial media warmed to 37°C was added directly to the flask to neutralize the 0.05% trypsin-EDTA. This mixture was triturated against the bottom of the flask to suspend any remaining cells and then removed from the flask entirely and placed in a 15ml Falcon tube. The cell suspension was then centrifuged at 1400 rpm for 5 mins. After centrifugation, the supernatant was removed and the pellet was resuspended in 1 ml of respiration buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l−1 fatty acid-free bovine serum albumin; pH 7.1). To determine the quantity of cells in the mixture, 10 μL was added and counted using a haemocytometer and a Zeiss Axioimager M2 (x10) while the rest of

the solution was kept on ice. After the concentration of cells in the solution was determined it was immediately used for high resolution respirometry measurements.

3.2.6 Mitochondrial Respiration and ROS Production in Permeabilized Cortical Astrocytes

To determine the respiration and ROS production rates of cortical astrocytes we performed high resolution respirometry. This was achieved by the suspension of a million primary cultured astrocytes in 2 ml of respiration buffer (in mM: 0.5 EGTA, 3 $MgCl_2$, 60 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose, 1 g l−1 fatty acid-free bovine serum albumin; pH 7.1) at a temperature of 37°C within an Oxygraph-2K (Oroboros Instruments, Innsbruck, Austria). Prior to stimulating respiration, the cells were permeabilized with 6 µM of digitonin. After 5-minutes of acclimation, substrates were added to stimulate the following respiration states in the permeabilized cells. For each addition of substrates 2-5 minutes was allowed for the stabilization of the respiration and H_2O_2 emission rates. Leak state respiration was stimulated with 20 mM glutamate (Sigma-Aldrich, St. Louis, MO, USA) and 5 mM malate (Sigma-Aldrich). Oxidative phosphorylation (Oxphos) respiration was stimulated with 20 mM ADP (Sigma-Aldrich), this initial stimulation provided oxygen consumption associated with complex I of the electron transport system, supplemented by the previously added glutamate and malate. Furthermore, additions of 10 mM pyruvate (Sigma-Aldrich) to maximally stimulate complex I, 20 mM of succinate (Sigma-Aldrich) to stimulate complex II, and 2 mM of ascorbate (Sigma-Aldrich) along with 0.5 mM of TMPD (Sigma-Aldrich) to stimulate complex IV were performed. Throughout these respiration states both oxygen consumption and H_2O_2 emission rates were measured, however, for stimulation of complex IV only oxygen consumption could be measured. Notably, all respiration and H_2O_2 emission rates were measured in acute normoxic conditions.

3.2.7 Statistical Analyses

Statistical analyses were conducted using GraphPad Prism 6, with an α level set to 0.05. Data are presented as mean ± SEM. N represents the number of independent culture preparations per condition. For individual comparisons, two-tailed unpaired t tests were performed and for multiple comparisons, two-way ANOVAs were performed with Tukey's multiple comparisons test as the post hoc.

3.3 Results

3.3.1 Mitochondrial Respiration of Cortical Astrocytes Grown in Different Oxidative Conditions

For this experiment, we grew isolated astrocytes in two different culture conditions: 21% oxygen (normoxia) and 3% oxygen (physiological hypoxia). As previously demonstrated, respiration of permeabilized cortical primary astrocytes in the *Fmr*1 KO mice when grown in normoxia was similar to that of WT mice (Fig. 8B-C). Consistent with findings presented in Chapter 2, the only notable difference in the normoxic condition was an increased leak state respiration in the *Fmr*1 KO astrocytes compared to WT ($F_{11,11}=4.425$; n=12 for WT; n=12 for KO; $P<0.05$ Fig. 8A). The importance of maintaining the physiological environment of the developing brain becomes clear when we look at the hypoxia condition. By measuring respiration of primary cortical astrocytes grown in physiological hypoxia, we found an increased leak and oxphos respiration in *Fmr*1 KO primary cortical astrocytes compared to WT ($F_{1,95}=6.995$; $P<0.05$; Fig. 8A-C). Not surprisingly, there was a significant main effect of respiration state ($F_{4,95}=258.3$; $P<0.0001$; Fig. 8A-C), indicating changes in respiration from state to state.

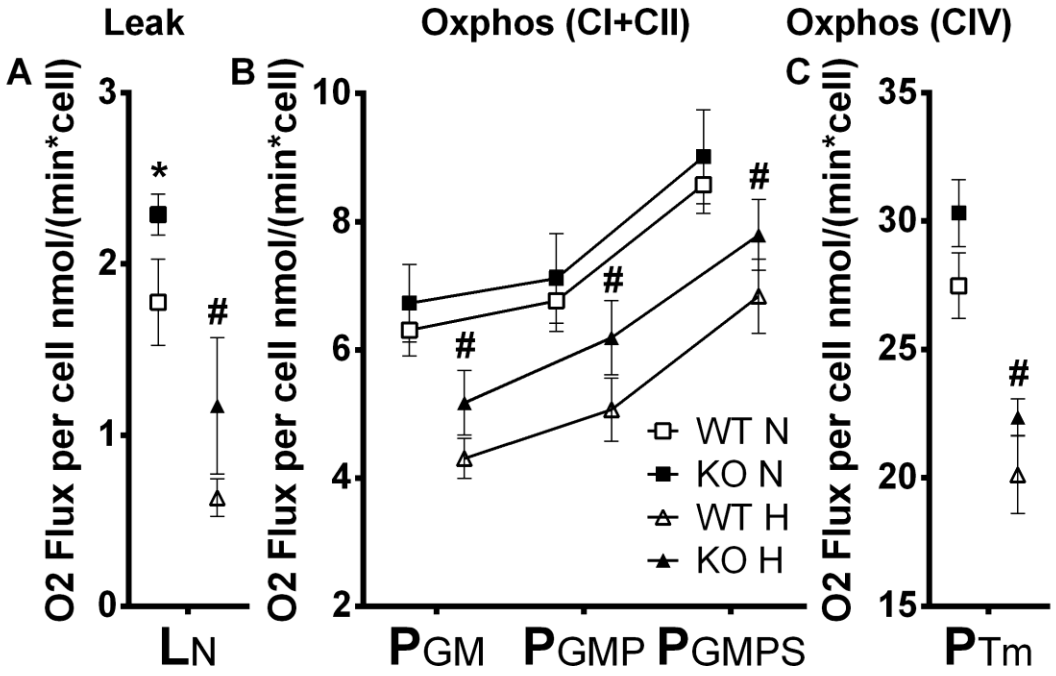

Figure 8. Respiration rates of WT (n=12 for normoxia; n=11 for hypoxia), and *Fmr*1 KO (n=12 for normoxia; n=10 for hypoxia) permeabilized primary cortical astrocytes were stimulated via sequential application of exogenous substrates: addition of glutamate and malate for leak respiration (L_N); then ADP (P_{GM}) for oxidative phosphorylation via complex I; pyruvate (PGMP) for maximal stimulation of complex I; succinate (P_{GMPS}) for stimulation of complex II; and finally, ascorbate and TMPD (P_{Tm}) for stimulation of complex IV. *Fmr*1 KO astrocytes grown in normoxia demonstrated significantly higher L_N values than WT (*A*), but no difference in oxidative phosphorylation capacity was measured (B *and C*). *Fmr*1 KO astrocytes grown in hypoxia demonstrated significantly higher L_N and OXPHOS values than WT (*A-C*). *-Significant effect, #- Significant main effect, $P<0.05$. Error bars, SEM.

3.3.2 ROS Production of Cortical Astrocytes Grown in Different Oxidative Conditions

Measurements of ROS production in permeabilized cortical astrocytes cultured in normoxia showed that ROS production was elevated in *Fmr*1 KO mice compared to WT mice (Fig. 9A). There was a significant main effect of genotype across all respiration states ($F_{1,72}=5.418$; n=9 for WT; n=11 for KO; $P=0.0227$; Fig. 9A) showing elevated ROS production during both leak state and OXPHOS respiration. Again, there was an expected significant effect of respiration state ($F_{3,72}=12.80$; $P < 0.0001$; Fig. 9A). Interestingly, when we measured the ROS production of permeabilized cortical astrocytes grown in hypoxia we found no differences in ROS production between the *Fmr*1 KO mice and the WT mice across all the respiration states (Fig. 9B). Despite the elevated ROS production, we discovered at normoxia, in hypoxia there seemed to be no alterations in the ROS production, or so we thought at first.

3.3.3 Sex Differences of Cortical Astrocyte Mitochondrial Respiration

Here, we analyzed the respiratory capacities and ROS production of *Fmr*1 KO astrocytes from both male and female mice. From primary astrocyte cultures maintained in normoxic conditions, there were no differences of oxygen comsumption in any of the respiration states between genotypes or sexes (Fig 10A-C). When comparing oxygen flux during respiration of primary astrocytes grown from female mice grown in hypoxia, again we did not observe differences between *Fmr*1 KO mice and WT mice (Fig 11A-C). However, when we analyzed astrocytes isolated from male mice in the same condition, we found both an increase of leak state respiration and an increase to OXPHOS respiration in *Fmr*1 KO mice compared to WT mice ($F_{1,40}=8.749$; $P<0.05$; Fig 11A-C). Thus, there were no differences in respiration capacity of either male or female astrocytes in normoxia, but in hypoxia there was elevated levels of respiration across all states in *Fmr*1 KO male mice compared to WT male mice.

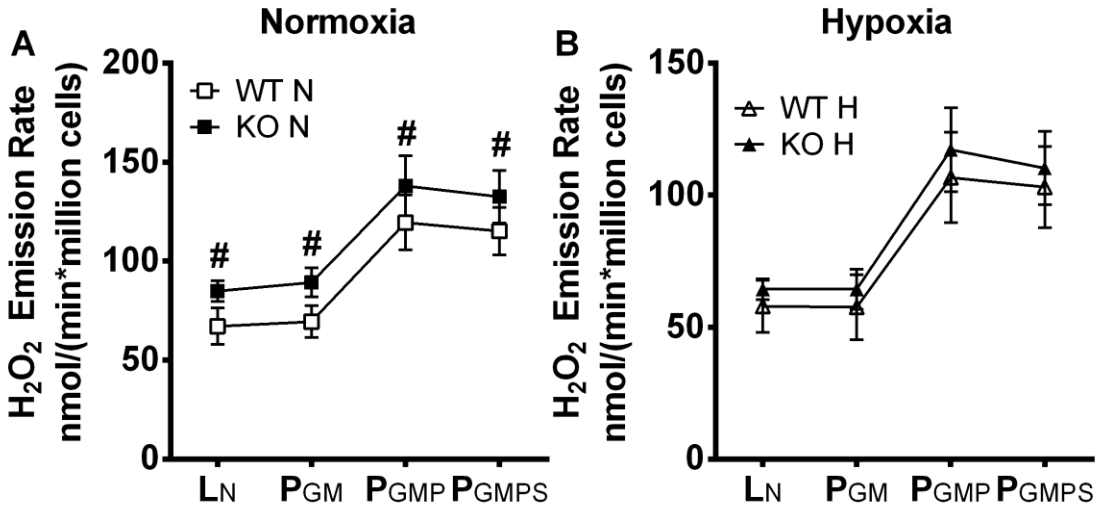

Figure 9. (*A-B*) H_2O_2 emission rates of WT (n=9 for normoxia; n=8 for hypoxia) and Fmr1 KO (n=11 for normoxia; n=9 for hypoxia) permeabilized primary cortical astrocytes. Respiration states were stimulated as previously stated: leak respiration (L_N) via glutamate and malate; oxidative phosphorylation of complex I with glutamate, malate, and ADP (P_{GM}); maximal stimulation of complex I with glutamate, malate, ADP, and pyruvate (P_{GMP}); and stimulation of complex II with glutamate, malate, ADP, pyruvate, and succinate (P_{GMPS}). *Fmr*1 KO astrocytes grown in normoxia demonstrated significantly higher H_2O_2 emission rates than WT (*A*), but no difference in emission rates was measured for astrocytes grown in hypoxia (B). #-Significant main effect, P<0.05. Error bars, SEM.

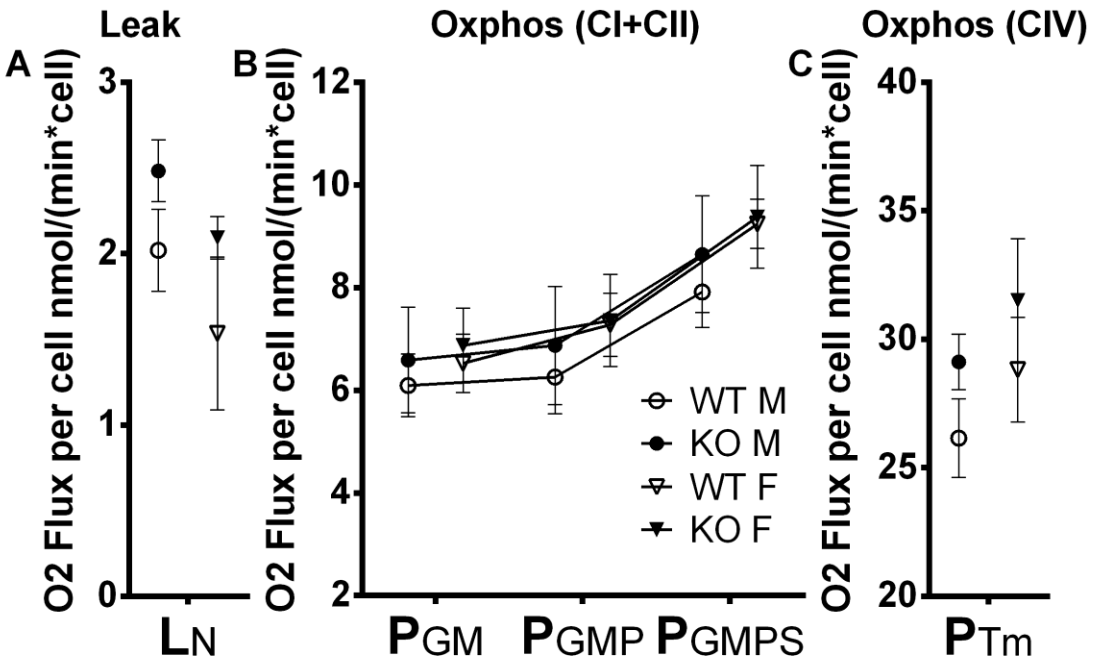

Figure 10. Respiration rates of WT (n=6 for male; n=6 for female), and *Fmr*1 KO (n=6 for male; n=6 for female) permeabilized primary cortical astrocytes grown in normoxia (21% O_2). Respiration states were stimulated as previously stated: leak respiration (L_N) via glutamate and malate; oxidative phosphorylation of complex I with glutamate, malate, and ADP (P_{GM}); maximal stimulation of complex I with glutamate, malate, ADP, and pyruvate (P_{GMP}); stimulation of complex II with glutamate, malate, ADP, pyruvate, and succinate (P_{GMPS}); and stimulation of complex IV with ascorbate and TMPD (P_{Tm}). There were no significnat effects. Error bars, SEM.

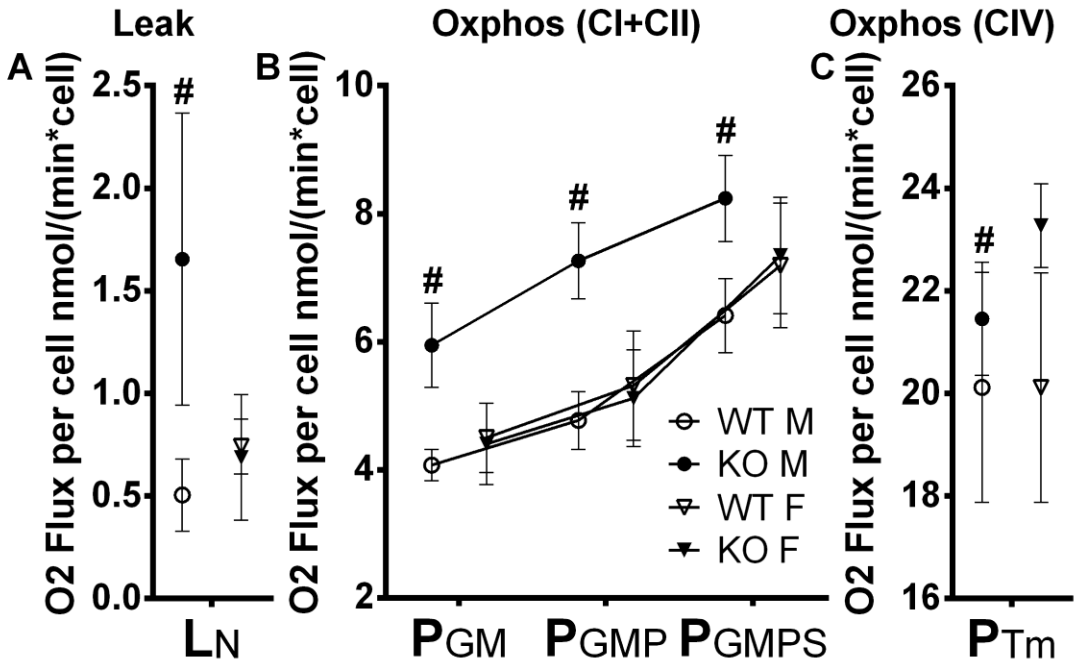

Figure 11. Respiration rates of WT (n=5 for male; n=6 for female), and *Fmr*1 KO (n=5 for male; n=5 for female) permeabilized primary cortical astrocytes grown in hypoxia (3% O_2). Respiration states were stimulated as previously stated: leak respiration (L_N) via glutamate and malate; oxidative phosphorylation of complex I with glutamate, malate, and ADP (P_{GM}); maximal stimulation of complex I with glutamate, malate, ADP, and pyruvate (P_{GMP}); stimulation of complex II with glutamate, malate, ADP, pyruvate, and succinate (P_{GMPS}); and stimulation of complex IV with ascorbate and TMPD (P_{Tm}). *Fmr*1 KO astrocytes collected from male mice demonstrated significantly higher L_N and oxphos values than WT (*A*-*C*). #- Significant main effect, P<0.05. Error bars, SEM.

3.3.4 Sex Differences of Cortical Astrocyte ROS Production

Lastly, we investigated sex differences associated with ROS production in various oxidative conditions within the context of FXS. As discovered in the previous study and confirmed here, there were elevated levels of ROS production in *Fmr*1 KO astrocytes compared to WT grown in a normoxic condition (Fig. 9A). When sex was accounted for, we found elevated ROS production in the astrocytes collected from male *Fmr*1 KO mice compared to those collected from male WT mice ($F_{1,36}=9.28$; $P<0.05$; Fig. 12A). However, we found no difference in ROS production for astrocytes collected from female *Fmr*1 KO mice compared to female WT mice (Fig. 12B). Due to this, it seems that ROS production in male astrocytes must account for the elevation of ROS production we observed in *Fmr*1 KO astrocytes when both sexes were pooled together ($F_{1,72}=5.418$; n=9 for WT; n=11 for KO; $P=0.0227$; Fig. 9A). In the hypoxia condition, when both sexes were pooled together there were no changes in ROS production (Fig. 9B). When we looked at males and females independently, we found a decreased ROS production of astrocytes collected from male *Fmr*1 KO mice compared to WT mice ($F_{1,24}=9.263$; $P<0.05$; Fig. 12C) but increased ROS production in astrocytes collected from female *Fmr*1 KO mice compared to female WT mice ($F_{1,28}=5.545$; $P<0.05$; Fig. 12D). The differential ROS production between sexes accounts for the consistent ROS production we discovered when sexes were pooled together in the hypoxic condition, as that result was an average of both male and female ROS production.

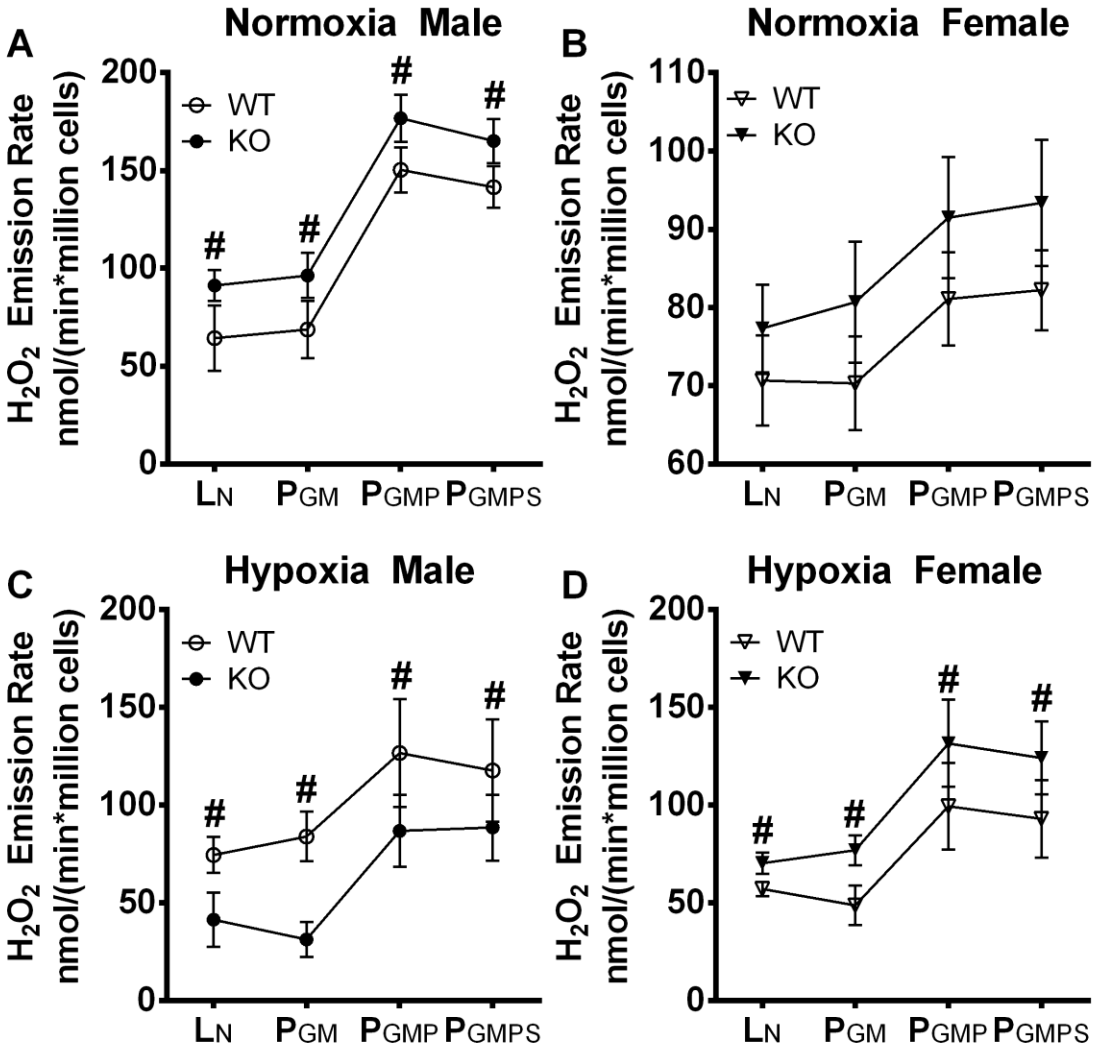

Figure 12. (*A-B*) H_2O_2 emission rates of WT (n=5 for male; n=4 for female), and *Fmr*1 KO (n=6 for male; n=5 for female) permeabilized cortical astrocytes grown in normoxic conditions.(*C-D*) H_2O_2 emission rates of WT (n=5 for male; n=6 for female), and Fmr1 KO (n=5 for male; n=5 for female) permeabilized cortical astrocytes grown in hypoxic conditions. Respiration states were stimulated as previously stated: leak respiration (L_N) via glutamate and malate; oxidative phosphorylation of complex I with glutamate, malate, and ADP (P_{GM}); maximal stimulation of complex I with glutamate, malate, ADP, and pyruvate (P_{GMP}); and stimulation of complex II with glutamate, malate, ADP, pyruvate, and succinate (P_{GMPS}). # - Significant main effect, $P<0.05$. Error bars, SEM.

3.4 Discussion

In this study, we demonstrated that culture growth conditions and sex are important determinants of mitochondrial function in astrocytes. By performing high resolution respirometry on permeabilized cultured astrocytes we were able to measure both oxygen consumption and hydrogen peroxide production at the cellular level. These measures allowed us to assess both the respiration and ROS production of astrocytes and compare across different oxidative conditions, sex, and disease. The genetic deletion of FMRP in FXS led to differential changes to both mitochondrial respiration and ROS production of astrocytes when culture conditions and sex were considered independently.

3.4.1 Impact of Physiological Hypoxia on Fmr1 KO Astrocyte Mitochondrial Bioenergetics

Across all respiration states there was an elevation of oxygen consumption in the *Fmr*1 KO astrocytes compared to the WT astrocytes when the cells were grown in physiological hypoxia (3% O_2). This was surprising since, when grown in normoxic conditions, there was an increase to mitochondrial respiration in *Fmr*1 KO astrocytes in leak state but not oxidative phosphorylation. This result suggests that in physiological conditions, it appears that astrocytes require higher levels of bioenergetics. This is in line with the hyperexcitability of the neural circuitry and the increased number of immature synapses found within the developing FXS brain (Contractor, Klyachko, & Portera-Cailliau, 2015). Given what astrocytes do to help in maturation of synapses and regulation of synaptic transmission, it is not surprising that demand and relative capacity of astrocytes is higher in FXS.

Increased mitochondrial complex activity has also been associated with oxidative stress in the brain (reviewed in Turrens, 2003). Previous studies have demonstrated the presence of oxidative stress markers within the FXS brain, including increased ROS levels and associated

oxidative damage in proteins and lipids found in the cortex later in life (el Bekay, et al., 2007; de Diego-Otero, et al., 2009). Elevated oxidative phosphorylation is also associated with increases in ROS production and, if not buffered appropriately, results in oxidative stress. Despite the higher levels of oxygen consumption in *Fmr*1 KO astrocytes in physiological hypoxia, there were no significant differences in ROS production when compared with WT astrocytes. This may have to do with the relatively low levels of oxygen in this condition and astrocytes ability to offset ROS production with reduction. Even though the oxygen consumption rate was higher in *Fmr*1 KO astrocytes compared to WT astrocytes in hypoxia, the oxygen consumption rates were significantly higher in both *Fmr*1 KO and WT astrocytes when grown in normoxia compared to WT astrocytes grown in hypoxia. It seems that in higher levels of oxygen and ROS production, the ability of the *Fmr*1 KO astrocytes to reduce free radicals is impaired.

Astrocytes protect themselves against the deleterious effects which occur in a restricted oxidative environment through a variety of mechanisms. One mechanism involves increased expression of hypoxia-inducible factor-1 (HIF-1), which promotes cell survival and has been found to protect astrocytes against oxidative damage (Chu et al., 2010). Specifically, a study performed by Badawi, Ramamoorthy, and Shi (2012) revealed that HIF-1 protects astrocytes against hypoxia-induced glutamate toxicity. Furthermore, HIFs have been found to increase glycolytic capacity and alter mitochondrial function (Papandreou et al., 2006). Taken together, these results suggest a potential role of HIF-1 in the altered mitochondrial bioenergetics uncovered in this study at physiological hypoxia. Along with the effects of HIF, astrocytes have been found to upregulate retinoid-related orphan receptor- α (RORα) when exposed to hypoxia (Jolly et al., 2011). RORα counteracts the deleterious effects of oxidative stress by increasing the expression of antioxidant proteins (Jolly et al., 2011). Additionally, activation of c-Myc is another astrocytic response to hypoxia which promotes cell survival and proliferation (Liu et al.,

2006). C-Myc promotes cell survival through inhibition of p21, a cyclin-dependent kinase inhibitor, and induction of cell cycle proteins which both allow the cell cycle process to occur (Liu et al., 2006). The increased leak state respiration and ROS production of *Fmr*1 KO astrocytes in hypoxia suggest there may be deficiencies involving these proteins which in turn affect the astrocytic response to hypoxia within FXS.

3.4.2 Sex Differences

Evaluating both sexes when assessing the oxidative respiration and ROS production of astrocytes is integral for understanding X-linked dominant disorders, which differentially affects males and females. Furthermore, sex differences also exist within the mitochondrial bioenergetics of astrocytes (Jaber et al, 2017). To evaluate this important variable, we analyzed both the oxygen consumption and ROS production data by comparing male derived *Fmr*1 KO astrocytes to male- derived WT astrocytes and evaluated how those differences related to comparisons made between female derived *Fmr*1 KO and WT astrocytes during different oxidative conditions. Firstly, when assessing the oxygen consumption for astrocytes grown in normoxia, we uncovered no differences in respiration within either sex associated with leak state or oxidative phosphorylation respiration. This result was consistent with previous findings that found that astrocytes grown in 21% O_2 showed no differences in bioenergetic parameters across male and female astrocytes (Jaber et al, 2017). In physiological hypoxia, we found an elevation in oxygen consumption across all respiration states of male derived *Fmr*1 KO astrocytes compared to male derived WT astrocytes. Despite this elevation in males there were no differences in respiration when comparing WT and *Fmr*1 KO astrocytes derived from female mice. This suggests that elevations in ROS production found in hypoxia-grown *Fmr*1 KO astrocytes was due only to male astrocytes and not female cells in the mixed cultures. While the

underlying cause of these sex differences emerging in these isolated cell preparations is not clear, there may be a role for differential sex hormone expression.

In females, estrogen is the primary sex hormone and is known to have neuroprotective roles within the brain. Estrogen influences the expression of SOD and GPx, both vital antioxidant enzymes that astrocytes express in high concentrations within the brain (Arnold & Beyer, 2009). Along with the presence of estrogen throughout the growth of the female-derived astrocytes, the activity of aromatase is higher in female astrocytes compared to male astrocytes (Liu et al., 2006). Aromatase is an enzyme responsible for converting testosterone to estradiol, which as mentioned earlier, provides neuroprotection (reviewed in Garcia-Segura et al., 2003). Therefore, higher levels of aromatase and the presence of estrogen supply females with increased neuroprotection. Whereas the male derived astrocytes are more prone to the deleterious effects of oxidative stress caused by the lack of FMRP. Furthermore, a study uncovered increased expression of the gene encoding for mitochondrial Complex I subunit Ndufa5 in astrocytes cultured in hypoxia (Chadwick et al., 2011). This change in expression may differentially influence the mitochondrial capacity between sexes and account for the increased respiration seen in *Fmr*1 KO males compared to WT males and not seen in *Fmr*1 KO females.

Lastly, we also assessed sex differences in ROS production of permeabilized cultured astrocytes grown in both normoxic and hypoxic conditions. To preface the discussion of sex differences in ROS production of cortical astrocytes we want to reiterate our previous findings when the analysis was performed prior to the consideration of sex. When we considered the effect of sex in normoxic conditions, we found that ROS production in male derived *Fmr*1 KO astrocytes was greater compared to male-derived WT astrocytes, but no difference in the ROS production between female derived *Fmr*1 KO astrocytes and female derived WT astrocytes.

Again, these discrepancies may be due to the neuroprotective effects of estrogen within the female derived astrocytes (Arnold & Beyer, 2009), making male cells may be more susceptible to disease conditions. Interestingly, male-derived astrocytes grown in the hypoxic conditions showed a relative reduction in the ROS production of *Fmr*1 KO astrocytes compared to WT. In contrast, female-derived astrocytes had elevated ROS production in *Fmr*1 KO astrocytes compared to WT when grown in hypoxic conditions. These findings present a puzzling paradox and suggest that inherent differences extend beyond sex hormones in these cells. One aspect of sexual dimorphism that has been left unmentioned within astrocytes is their response to inflammation. Santos-Galindo et al (2011) found that females increase expression of interferon-inducible protein 10, whereas males increase expression of IL6, TNFα, and IL1β in the inflammatory response to lipopolysaccharide. The presence of these sex differences supports the notion that they are more considerations beyond the effect of sex hormones when discussing cellular stress.

It is important to note that the sex differences we are investigating within this study are solely influenced by the sex of the mice and not by differential expression of FMRP. Clinically, males are disproportionately affected by this disorder and typically present with more severe symptoms. This is explained through the inheritance pattern of FXS. Since it is an X-linked dominant disorder, males with the full mutation experience complete silencing of the *Fmr*1 gene. However, since females experience mosaicism and X-inactivation the severity of the disorder depends of the expression levels of FMRP. In our study, the *Fmr*1 KO mouse model has complete silencing of the *Fmr*1 gene in both males and females and because of this the differences cannot be attributed to differential FMRP expression. Thus, in future studies the use of induced or partial knockdown of the Fmr1 gene would be desirable to further our understanding of true sex differences in this disease. In any case, our findings of sex differences

within the mitochondrial bioenergetics of astrocytes within the context of FXS supports the notion that sex is a prominent determinant for mitochondrial function and pathologies (reviewed in Ventura-Clapier et al., 2017).

Chapter 4: Discussion

Demonstrated in the previous chapters of this thesis are important findings related to the pathology of FXS, the most common heritable cause of intellectual disability. This thesis and the resulting publications will contribute to the scientific community's understanding of FXS pathology which will guide future studies in further elucidation of the pathology and in developing clinical treatments for this disease. Using the *Fmr*1 KO mouse model we addressed all of our research objectives. I characterized the mitochondrial respiration and ROS production of *Fmr*1 KO astrocytes, I assessed the expression of two isoforms of NADPH-oxidase, a common source of ROS production, and I evaluated the expression of key antioxidant enzymes. Furthermore, we investigated the effects of sex and physiological hypoxia on the mitochondrial bioenergetics of astrocytes. Ultimately, this investigation evaluated various sources of oxidative stress within *Fmr*1 KO astrocytes and cortical tissue including mitochondrial dysfunction, altered NADPH-oxidase expression, and altered antioxidant enzyme expression.

4.1 Mitochondrial Dysfunction

My first objective in this project was to characterize the mitochondrial respiration and ROS production of *Fmr*1 KO astrocytes. This was accomplished through the performance of high resolution respirometry on primary cultured astrocytes. Ultimately, these measures assessed the presence of oxidative imbalances and evaluated for dysfunction in mitochondrial oxidative phosphorylation. Mitochondrial dysfunction (MD) includes a variety of deficits all leading to inefficient energy production and in many cases, disease. MD has been associated with many neurodevelopmental disorders and recently has been directly linked with FXS (Shen et al, 2019). In previous studies, FMRP has been found to influence microtubule network formation and axonal transport of mitochondria in drosophila (Yao et al., 2011). Improper transport of

mitochondria throughout the axon may play a part in the pathogenesis of FXS. Furthermore, increased levels of ROS production, lipid peroxidation, and protein oxidation have been found within the FXS brain, potentially as a result of MD (el Bekay et al., 2007). Along with these indirect findings of MD within FXS, a previous study conducted by Shen et al (2019) brought to light direct evidence. They uncovered dysfunctions in mitochondrial fusion and dendritic maturation associated with the loss of FMRP in hippocampal neurons. Additionally, they found increased oxidative stress in hippocampal neurons through the presence of elevated levels of superoxide, a common free radical.

Based on the presence of oxidative imbalances in both neurons and brain tissue, along with evidence of MD, we expected to find increased mitochondrial respiration and ROS production within *Fmr*1 KO astrocytes. We expected these increases since astrocytes are integral regulators of CNS oxidative homeostasis and they widely express FMRP throughout neurodevelopment (reviewed in Till, 2010). From our experiments we discovered elevations of leak state respiration and increased production of ROS in *Fmr*1 KO astrocytes compared to WT astrocytes. These findings aligned with previous literature and with our predictions for astrocyte mitochondrial bioenergetics within FXS. While we found no change in respiration during OXPHOS, when we only considered male populations of KO and WT mice, we did uncover elevations of OXPHOS respiration in *Fmr*1 KO astrocytes. Based on our results, mitochondrial abnormalities in respiration and ROS production of astrocytes play a role in FXS pathology. Further investigations on the impact astrocytes have on neurons and neuronal development needs to be evaluated to fully understand how our results fit within the pathology of FXS.

4.2 NADPH-Oxidase (NOX)

Along with MD, another common source of oxidative imbalance and free radical production within the brain is increased NADPH-oxidase (NOX) activity. This source of ROS production has been associated with many neurodevelopment disorders and specifically, increased NOX activation has been discovered in *Fmr*1 KO brain tissue (el Bekay et al., 2009). Along with this, crosstalk between the mitochondria and NOX results in the activation of NOX during increased ROS production from mitochondria, which would perpetuate the production of ROS within the cell (reviewed in Jiang et al., 2011). Furthermore, Rac1, a subunit of NOX, has been found to be influenced by FMRP and increases in Rac1 stimulation are directly correlated with increased NOX activity (el Bekay et al., 2007). In our investigations we assessed expression levels of NOX2 and NOX4, which are the most abundant isoforms of NOX within the CNS.

Interestingly, we discovered an elevation of NOX2 in *Fmr*1 KO astrocytes along with no differences in expression within cortical tissue. The elevation of NOX2 in *Fmr*1 KO astrocytes suggest a role of NOX within FXS pathology. Upregulation of NOX2 has been associated with Alzheimer's disease in response to increased beta-amyloid accumulation, a type of cellular stress (Chay et al., 2017). Furthermore, previous studies have indicated increased activity of NOX within the FXS brain (el Bekay, et al., 2007). Unlike NOX2, when NOX4 expression was evaluated, no genotypic differences were found across *Fmr*1 KO and WT astrocytes. However, there was a reduction of NOX4 expression in *Fmr*1 KO cortical tissue. It is known that NOX4 is only slightly expressed in astrocytes and in contrast to SOD2, is downregulated by increased levels of ATP (Gray et al., 2015; Shanmugasundaram et al., 2017). It would be reasonable to suggest that the downregulation of NOX4 in *Fmr*1 KO cortical tissue is a result of increased ATP production occurring within the FXS brain. Despite these results indicating dysregulations

of NOX, future studies must be completed to understand the activation of NOX2 and NOX4 within FXS and how they contribute to ROS production.

4.3 Antioxidant Defence System

4.3.1 Catalase (CAT)

The system in place to combat increased oxidative stress within the CNS is the antioxidant defence system. Impairments to this defence may reduce the ability for it to buffer oxidative stress, resulting in increased damage. Since astrocytes are the main provider of antioxidants within the CNS, they play a very important role in buffering oxidative imbalances (Wilson, 1997). The first enzyme we investigated was catalase (CAT), an enzyme responsible for the conversion of hydrogen peroxide to water and oxygen in the mitochondria and the cytosol, disabling the reactive properties of hydrogen peroxide. When CAT expression levels were measured, we uncovered increased CAT expression in *Fmr*1 KO astrocytes and no associated changes in CAT expression in *Fmr*1 KO cortical tissue. Antioxidant expression typically increases in response to oxidative stress and more specifically CAT expression has been found to increase in response to hydrogen peroxide induced stress (Hunt et al., 1998). Therefore, it is possible that the associated increase in expression is a response to the increased hydrogen peroxide emissions I found in *Fmr*1 KO astrocytes. As for the expression level of CAT in cortical tissue, the lack of difference between genotypes can be explained by the presence of other cell types within the cortex which may express CAT at different levels and shadow the difference seen in *Fmr*1 KO cortical astrocytes.

4.3.2 Superoxide Dismutase (SOD)

Another key antioxidant enzyme within astrocytes is superoxide dismutase (SOD). SOD is an enzyme responsible for converting superoxide into hydrogen peroxide, a vital step in the antioxidant defence. Two isoforms of SOD exist within the brain, SOD1 and SOD2, also known as cytosolic and mitochondrial SOD, respectively. Interestingly, Bechara et al (2009) discovered that FMRP plays a role in regulating expression levels of SOD1, and that the lack of FMRP results in decreased expression of SOD1 in brain tissue. They proposed that the dysregulation of SOD1 may play a role in FXS pathology. However, through our experiments, we found no significant differences between SOD1 or SOD2 expression in *Fmr*1 KO astrocytes and cortical tissue compared to WT. Our results suggest that SOD expression is not altered or regulated by FMRP. However, increased ROS production within *Fmr*1 KO astrocytes may perpetuate oxidative damage with no associated increase in SOD expression. To elaborate, since there is evidence of increased ROS and no alterations in SOD expression, this may suggest a deficient response in the antioxidant defence. Furthermore, although expression of SOD was not altered, activity of this enzyme remained unevaluated.

4.3.3 Glutathione Cycle Enzymes

The last component of the antioxidant system we investigated was the glutathione cycle enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR). El Bekay et al (2007) had previously found deficits to the glutathione cycle within the FXS brain. However, these results were found within brain tissue and investigation into the astrocytic glutathione cycle defence remained untapped within FXS. Due to the presence of increased ROS production we uncovered in *Fmr*1 KO astrocytes, along with the alterations of the glutathione cycle discovered, we expected decreased expression of both GPx and GR. Interestingly, in our investigations we discovered no differences in expression of GPX or GR in *Fmr*1 KO astrocytes and cortical tissue

compared to WT. Based on these results we would not suggest that FMRP regulates the expression of these enzymes.

Since the expression of most of the antioxidant enzymes we investigated were similar across genotypes our results do not suggest that a lack of antioxidants would be contributing to the oxidative imbalances seen within FXS. Contradictory to our predictions we found increased CAT expression within *Fmr*1 KO astrocytes likely as a response to the increased hydrogen peroxide emissions. Since previous studies indicated oxidative stress within the FXS brain, and my results show increased ROS production with Fmr1 KO astrocytes, it may be that the antioxidant defence system is deficient in its response to ROS production. To determine if this is in fact true, more studies need to investigate both expression and activity of the antioxidant defence of Fmr1 KO astrocytes in response to increased ROS production.

4.4 Other Potential Sources of Oxidative Stress

4.4.1 Glutamate Toxicity

Within this thesis, we investigated mitochondrial dysfunction, NADPH-oxidase expression, and the antioxidant system contributors to the oxidative imbalances within FXS pathology. However, outside of these parameters are other sources which may be contributing to the oxidative stress observed in this and previous studies of FXS. One common source of ROS production, which was left uninvestigated, is neurotoxicity caused by elevated levels of glutamate in the brain. Previous studies elucidated the effects that elevations in glutamate have on neuronal development within FXS and astrocytes have been known to regulate levels of glutamate at the synapse (Mahmoud et al 2019). In particular, Wang et al (2016) discovered that neurotoxicity within the FXS brain can be stimulated by improper regulation of glutamate and γ-aminobutyric acid (GABA) by *Fmr*1 KO astrocytes. Furthermore, this neurotoxicity was directly

linked to oxidative stress in cultured neurons (Wang et al., 2016). These results suggest that improper glutamate regulation by astrocytes contributes, at least in part, to the oxidative stress in the FXS brain. Based on this it seems that the oxidative stress occurring within FXS is multifactorial, and the lack of FMRP triggers multiple molecular sources leading to the production of ROS.

4.4.2 Beta-Amyloid Accumulation

Another source of oxidative stress which may be occurring within FXS is the accumulation of the protein beta-amyloid (Aβ). Westmark & Malter (2007) found increased levels of amyloid precursor protein (APP) in *Fmr*1 KO mice and proposed that the accumulation of this protein led to synaptic loss, impaired neurotransmission, and ultimately oxidative stress in the FXS brain. Beta-amyloid accumulation has also been found to play a role in Alzheimer's disease, which along with FXS, is associated with oxidative stress in the brain (Butterfield, 2003). Furthermore, evidence suggests APP is important for the formation of synapses within the developing brain (Akaaboune et al., 2000). The link between Aβ and oxidative stress needs to be evaluated further within FXS pathology to fully understand the impacts on neurodevelopment.

4.4.3 Receptor for Advanced Glycation End-products (RAGE)

When discussing oxidative stress in the brain it is important to consider the receptor for advanced glycation end-products (RAGE), a transmembrane receptor highly expressed throughout the CNS and in both neurons and astrocytes (Derk et al, 2018). RAGE acts as a modulator of inflammation within many organs throughout the body. Specifically, RAGE has been implicated with regulating inflammation within the CNS and increased expression has been linked with ROS production, neurological disease, and hypoxia (Walker et al., 2015; Xu et al., 2010). Once a RAGE ligand binds to RAGE, there is a signalling cascade leading to the

production of ROS, increased inflammation, and cell migration (Derk et al., 2018). The production of ROS associated with RAGE mainly occurs through activation of NOX, which as mentioned earlier, is increased in the FXS brain (Piras et at., 2016; El Bekay et al., 2007). Along with this, increased RAGE activity has been linked to many neuropathies including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis (reviewed in Stephenson et al., 2018). Despite the connection between RAGE, NOX activation, and oxidative stress, this signaling pathway has yet to be linked with FXS. There have not been any studies investigating the RAGE signalling pathway within FXS. Further evaluations of the effects of altered RAGE within the FXS brain may offer additional insights into causes of oxidative imbalance in the FXS brain.

4.4.4 Fenton Reaction

When there is overproduction of free radicals and inadequate antioxidant defence many detrimental effects can occur. These include DNA damage, lipid peroxidation, and protein oxidation. Normally, the antioxidant system is capable of buffering these reactive species, however in some cases the capacity of the system is overwhelmed. One aspect of free radical production that has remained unmentioned thus far is the Fenton reaction. The Fenton reaction is a reaction between free iron and hydrogen peroxide resulting in the production of the highly reactive hydroxyl radical (reviewed in Lipinski, 2011). This is particularly concerning since increased levels of hydroxyl radicals are directly linked with increased oxidative damage. Therefore, alterations to this reaction can influence oxidative damage and stress. Specifically, when there are increased levels of free iron and increased hydrogen peroxide the Fenton reaction can lead to neuronal cell death (Lu et al., 2015). However, neuronal cell death has not been noted within FXS during development and actually, an aspect of FXS pathology is impaired cell death in the developing brain (Cheng, Corbin, & Levy, 2013). Therefore, it is unlikely this reaction is

involved with the oxidative imbalances found in this thesis. However, our results did indicate increased hydrogen peroxide emissions in *Fmr*1 KO astrocytes. Further investigations ought to be completed on the levels of free iron and frequency of the Fenton reaction to determine if this concerning reaction is contributing to oxidative imbalances within FXS.

4.5 Physiological Hypoxia

While the vast majority of all tissue culture preparations are incubated in normoxia, there is a growing demand within the developmental neuroscience field to also include conditions of physiological hypoxia. The brain develops in oxidative conditions between 1-8% O_2, much different from atmospheric oxygen conditions (Ikonomidou & Kaindl, 2011). With this in mind, we prepared primary astrocyte cultures in both physiologically relevant hypoxia (3% O_2) and the traditional culture methodology using atmospheric oxygen levels (21% O_2). Oxidative conditions are particularly important when investigating mitochondrial respiration and ROS production, since these parameters are directly influenced by the availability of oxygen. Furthermore, Jaber et al (2017) supported the investigation of astrocytes at physiological oxygen tensions through the discovery of sex differences in astrocytic mitochondrial bioenergetics that were present in physiological hypoxia but not present in normoxia. Our findings also supported the use of physiologically relevant oxidative conditions for neural cell cultures.

When astrocytes were grown in physiological hypoxia (3% O_2) we found both genotypic and sex differences that were not observed in normoxia culture preparations. Specifically, we found elevations of both leak state and OXPHOS respiration in *Fmr*1 KO astrocytes compared to WT. This result suggests that in physiological conditions, *Fmr*1 KO astrocytes require higher levels of bioenergetics than WT, indicating increased activity. Astrocytes play an integral role in synaptogenesis and the increased activity may be associated with the greater number of

immature synapses and hyperexcitability found within the FXS cortex (Contractor, Klyachko, & Portera-Cailliau, 2016). Along with these findings we found discrepancies between respiration and ROS production between male and female astrocytes across the different oxidative conditions. Taken together, the use of physiological hypoxia was a vital component to this experimental design and should be considered for future studies of neural cell types.

How the astrocytic response to hypoxia is affected by the lack of FMRP is an important aspect to consider within this thesis. Astrocytes respond to hypoxia by increasing expression of HIF-1 and RORα (Chu et al., 2010; Jolly et al., 2011). Both these proteins have been found to protect astrocytes from oxidative damage and specifically, RORα has been found to increase expression of antioxidant proteins (Jolly et al., 2011). In conjunction with my results indicating increased ROS production in *Fmr*1 KO astrocytes at physiological hypoxia, it is plausible that there is a differential response to hypoxia involving HIF-1 and/or RORα across genotypes. Additionally, astrocytes respond to ROS through activation of c-Myc, another protein involved in cell proliferation (Liu et al., 2006). Due to this, c-Myc activation may also be differentially affected by the lack of FMRP in its response to physiological hypoxia.

4.6 Sex Differences

To address my last objective mitochondrial respiration and ROS production of *Fmr*1 KO and WT astrocytes were analyzed across sexes. Being an X-linked dominant disorder FXS differentially affects males and females, making sex differences an important aspect to consider. Furthermore, estrogen has a neuroprotective role and improves the antioxidant defence in females by increasing expression of SOD and GPx (Arnold & Beyer, 2009). Along with these discrepancies, Jaber et al (2017) uncovered a higher maximal respiratory capacity for male astrocytes compared to female astrocytes, indicating sex differences in the mitochondrial

bioenergetics of astrocytes. Based on previous studies we predicted females to be less affected by the deleterious affects of oxidative stress due to their increased neuroprotection. This prediction was supported through our discovery of elevated respiration and ROS production in *Fmr*1 KO male-derived astrocytes with no associated increases in female-derived astrocytes. Estrogen provides neuroprotection in females by influencing the expression of SOD and GPx, both of which are heavily expressed in astrocytes (Arnold & Beyer, 2009). Therefore, it seems that differential sex hormone expression impacts the mitochondrial bioenergetics of *Fmr*1 KO astrocytes. Taken together, our results corroborate the idea that sex is an integral component when investigating mitochondrial function within disease.

4.7 Experimental Considerations

4.7.1 Permeabilized cell preparations for high resolution respirometry

In the present thesis, permeabilized cell preparations were measured by high resolution respirometry for the determination of mitochondrial respiration and ROS production. This methodology was chosen as it allowed the separation of individual mitochondrial complex respiratory capacities and simultaneous measurements of hydrogen peroxide emissions. In order to achieve this the cell membrane of primary cultured astrocytes was permeabilized with digitonin. This permeabilization causes the removal of cytosolic solutes, reducing the physiological relevance of these measurements. Furthermore, permeabilization may also lead to damage to the outer mitochondrial membrane. Intact cells provide another method for measuring respiration. This method allows for the measurement of basal, coupled, and uncoupled respiration and provides a clearer understanding of the maximal mitochondrial electron transport capacity (Djafarzadeh & Jakob, 2017). Ultimately, intact cells are more physiologically relevant since the cellular environment is not affected by permeabilization and the mitochondria are

undisturbed. However, despite the advantages, intact cell preparations do not allow for measurements of ROS production when using high resolution respirometry, and for this reason alone, permeabilized cell preparations were chosen.

4.7.2 Total Protein Normalization for Western Blots

Traditionally, western blot analysis is performed with normalization to a house keeping protein loading control. House keeping proteins are single proteins found ubiquitously in high abundance across preparations. For this reason, normalization to the antibody-based immunodetection measurements of a house keeping protein has been used for western blots. However, previous studies have indicated that these loading controls do not accurately reflect differences in protein concentration (Aldridge et al., 2008). Furthermore, immunodetection of house keeping proteins is more prone to oversaturation than total protein measurements (Aldridge et al., 2008). Therefore, for western blot analysis performed within this thesis I used stain-free imaging of total protein. This method uses a proprietary trihalo compound which, after UV activation, binds to tryptophan residues within the samples and increases the natural fluorescence of proteins (Gürtler et al., 2013). This allows for the determination of total protein content within each lane and a reliable measurement for normalization.

4.8 Future Directions

4.8.1 Live Imaging to Assess Mitochondrial Dynamics and ROS Production

One aspect of mitochondrial dysfunction that remains uninvestigated within *Fmr*1 KO astrocytes is mitochondrial fragmentation, and specifically, mitochondrial fusion and fission events. These potential deficits could be assessed through live fluorescent imaging. MitoTracker Red CMXRos provides a stain for mitochondria that would allow for determination of mitochondrial morphology, including size and fragmentation. Shen et al (2019) investigated

these parameters within *Fmr*1 KO hippocampal neurons, uncovering mitochondrial fragmentation and mitochondrial fusion deficits. Since astrocytes are integral for CNS homeostasis it is important to understand all aspects of mitochondrial function and potential dysfunction within FXS. Based on this, a valuable experiment to further the work completed in this thesis is the evaluation of astrocyte mitochondrial dynamics within FXS.

Along with measurements of mitochondrial dynamics it would be valuable to assess the cause of ROS production we measured within *Fmr*1 KO astrocytes. There are many potential sources of ROS production within FXS as discussed previously. However, using a ROS dye, and a fluorescent live imaging set up, this would allow exogenous perturbation of astrocytes with particular antagonists, agonists and substrates. In this way we could assess molecular pathways involved with ROS production and measure their effects on *Fmr*1 KO astrocytes compared to WT astrocytes.

4.8.2 Co-cultures of Astrocytes and Neurons

When evaluating the role of astrocytes within a pathology it is important to consider the astrocytic effect on neurons and neuronal development. One method of evaluating astrocytic contributions is by growing both astrocytes and neurons in co-culture. Through this experimental design we can see how the loss of FMRP effects the astrocytes role in synaptogenesis. This method of culturing allows for growth of astrocytes and neurons prepared from different mice, meaning astrocytes from one genotype can be grown with neurons from a different genotype. With this in mind, growing *Fmr*1 KO astrocytes in the presence of WT neurons would allow us to directly assess how the extra metabolic demand neurons require of astrocytes influences their susceptibility to oxidative stress. Furthermore, using co-cultures, we would be able to assess the effects of both male and female *Fmr*1 KO astrocytes on WT neurons. With sex pairing across the

cell types, this would allow us to determine the effect that sex hormones have on astrocyte mediated oxidative homeostasis within FXS, providing more support for the notion that sex plays an integral role in neuropathies.

4.8.3 Enzyme Activities

To address the second objective of this thesis, western blots were performed to determine the expression of key antioxidant enzymes. However, despite the expression levels of these enzymes they may have differential activity across genotypes. Measuring enzyme activity could provide a valuable insight into how FMRP affects these proteins. Specifically, performing assays to measure the activity of CAT, SOD1, and SOD2 would provide further support of our findings. Furthermore, the most reliable method of assessing the glutathione cycle within samples is to measure the ratios of reduced (GSH) and oxidized glutathione (GSSG), the ratios of these products would provide a clear picture of both GPx and GR capacity (Owen and Butterfield, 2010). Along with assessing the activities of antioxidant enzymes, it would be valuable to measure the activity of NOX. This would allow for appropriate determination of NOX activation, and further support that NOX is linked with the ROS production we determined in *Fmr*1 KO astrocytes.

4.8.4 Measurements of Brain tissue and Astrocytes after MACS

Performing high resolution respirometry on brain tissue samples and astrocytes after magnetic-activated cell sorting (MACS) would provide an important overall assessment of the pathophysiology within the FXS brain. Furthermore, the use of these methods would allow for measurements of various brain regions and developmental time points. Due to the restrictions of cell culture preparations these aspects could not be considered in cultured astrocytes. However, the investigations within brain tissue and astrocytes isolated from brain tissue could provide

invaluable information for the pathology of FXS. The opportunity to perform high resolution respirometry on brain tissue and freshly isolated astrocytes would allow for the investigation of both mitochondrial respiration and ROS production in the cortex, hippocampus, striatum, and cerebellum, all of which have been found to highly express FMRP throughout development (Arsenault et al., 2016). Furthermore, these measurements could be performed at various developmental time points such as P1, P7, P14, and P21. These time points are specific to the peak and end of both neurogenesis (P1-P14) and gliogenesis (P7-P21) (Reemst et al, 2016). These investigations would give a comprehensive analysis of mitochondrial function in *Fmr*1 KO astrocytes and the FXS brain across neurodevelopment.

Along with the physiological measurements described above, it would be valuable to assess the antioxidant system across different brain regions and throughout neurodevelopment. By performing western blots on brain tissue from the cortex, hippocampus, striatum, and cerebellum we could gather a picture of the antioxidant defence throughout the FXS brain. These particular brain regions are of interest since they exhibit immature dendritic spines due to the absence of FMRP (Ellegood et al., 2010). Furthermore, if we investigate the expression levels of the key antioxidant enzymes at P1, P7, P14, and P21, we could assess the antioxidant system defence throughout development of the FXS brain. Similar measurements could also be performed on NOX to determine its expression throughout the brain and across neurodevelopment.

4.9 Conclusions

Overall, this thesis examined possible astrocytic deficits in oxidative homeostasis within FXS. We uncovered increased leak state respiration, increased ROS production, increased NOX expression and sex differences all within *Fmr*1 KO astrocytes. From our findings we

revealed novel contributors to FXS pathology and more insight into the role of astrocytes in the disease. Additionally, our findings revealed the importance of considering differences in cellular culture preparations and the importance of investigating sex differences within diseases associated with mitochondrial abnormalities. The present thesis provides a stepping-stone within the pond of neurodevelopmental pathologies and the findings within this thesis may contribute to the development of a treatment for FXS and further studies of neurodevelopmental disorders.

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