Alterations in Neural Connectivity following Placental Insufficiency, its Role in Neurodevelopmental Disabilities, and the Neuropreventive Effects of Broccoli Sprout Supplementation: A Potential Therapeutic Intervention

by

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Abstract

Background

Intrauterine growth restriction (IUGR) as a result of suboptimal *in utero* environments can cause perinatal brain injury and ultimately lead to neurodevelopmental disorders (NDD). In the developed world placental insufficiency (PI) is the leading cause of IUGR. This antepartum insult results in ischemic injury, oxidative stress, and chronic sequelae of fetal brain damage that can alter neuron development. As antepartum insults cause more than 70% of perinatal brain injury, targeting this vulnerable fetal population with a novel neuropreventive therapy can ideally reduce the severity and incidence of NDD. An established model of PI was used to explore the possible neuropreventive benefits of a natural health product; broccoli sprouts (BrSp), to treat this critical window of brain development currently without therapeutic interventions.

Methods

Bilateral uterine artery ligation as a model of PI was induced on day 20 of a 23-day gestation in the pregnant rat dam. Dams were randomly allocated to receive 200mg of BrSp daily from gestational day 15 until postnatal day (P) 21. On P35 animals were euthanized and brain tissue was extracted, fixed, and processed for Golgi-Cox analysis. Neurons were traced using a camera lucida and underwent Sholl analysis for dendritic length (DL), branch point analysis (BPs) for dendritic complexity, and spine density (SD) analysis as a measure of synapses.

Results

Our results indicate that BrSp afford neuropreventive benefits in neuronal morphology of the CA1 region of the hippocampus, specifically in the basal dendrites. This was demonstrated by reduced DL and BPs in the basal dendrites of IUGR animals that was prevented with the supplementation of BrSp. Additionally, male IUGR offspring were shown to be more significantly affected than IUGR females. Morphological assessment of the neurons in the primary motor cortex did not show an effect of IUGR, however BrSp increased both DL and BPs. This demonstrates the potential nutritive benefits of maternal BrSp consumption on the fetal brain. However, further studies will be needed to determine the effects of BrSp on the function of neurons, and various other brain regions.

Conclusion

The results of this study show that BrSp supplemented during late gestation and lactation can prevent alterations to the neuron caused by PI induced IUGR. These changes may contribute to the behavioural changes previously seen in this model. This suggests BrSp is an effective and novel neuropreventive approach to NDD associated with antepartum insults and perinatal brain injury.

Preface

This thesis is an original work by Ashley Margaret Anne Bahry. The University of Alberta Research Ethics Board: Animal Care and Use Committee Health Sciences granted ethics approval for this research, “Natural health products protect the newborn brain from injury”, No. AUP00000364, 7/20/2016.

Dedication

To Ross,

His love, optimism, and unwavering support

made it possible for me to complete this journey.

To my Parents,

Who taught me the most important lessons in life.

To my Grandparents,

From whom I get my stubbornness and strength.

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List of Abbreviations

ANOVA- Analysis of Variance

AP(s)- Action Potential(s)

ARE- Antioxidant Response Element

ATP- Adenosine Triphosphate

BPs- Branch Points

BrSp- Broccoli Sprouts

BUAL- Bilateral Uterine Artery Ligation

Ca^{2+}- Calcium

CP- Cerebral Palsy

Cys- Cysteine

DL- Dendritic Length

GD- Gestational Day

GSH- Glutathione

IUGR- Intrauterine Growth Restriction

Keap1- Kelch-like ECH-associated Protein 1

MRI- Magnetic resonance imaging

M1- Primary Motor Cortex

NDD- Neurodevelopmental Disorders

Nrf2- Nuclear Factor E2-related Factor 2

NT(s)- Neurotransmitter(s)

OL(s)- Oligodendrocyte(s)

OS- Oxidative Stress

P- Postnatal Day

PI- Placental Insufficiency

PN(s)- Pyramidal Neuron(s)

ROS- Reactive Oxygen Species

SEM- Standard Error of the Mean

SFN- Sulforaphane

1 INTRODUCTION

1.1 Neurodevelopmental Disorders

Neurodevelopmental disorders (NDD) are common, with a childhood prevalence of 13-14% worldwide and a major impact not only on the child but caregiver(s) health. [1,2] The Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition defines NDD as "*a group of conditions with onset in the developmental period. These disorders typically manifest early in development, often before the child enters grade school, and are characterized by developmental deficits that produce impairments of personal, social, academic, or occupational functioning…*". [3] As chronic conditions, NDD inflict significant emotional and economic burdens on the individual, their families and society. [2,4-6] It has been shown that NDD have a detrimental impact on school achievement and health of children as reported by school attendance, hospitalizations, and medication use. [2,4] In addition to these findings, caregiver(s) well-being has been shown to depend on that of their children. Nursing a child with NDD increases depressive symptoms, family distress, and caregiver(s) odds of a chronic condition or activity limitation. [6] Diagnosis and treatment of these disorders can be difficult and are further complicated by the fact that NDD can co-occur, increasing the severity of individual conditions. [2-4]

Causes of perinatal brain injury resulting in NDD are multifactorial and can arise during antepartum, intrapartum, or postpartum periods. Intrapartum hypoxia attributes to about 4% of perinatal brain injury while antepartum insults are responsible for approximately 70-90%. [7] Thus, injury during the antepartum period contributes to the majority of perinatal brain injury and plays a crucial role in subsequent development of NDD. Intrauterine growth restriction (IUGR) is one

such antepartum risk factor reported to increase hypoxic-ischemic encephalopathy by 40 fold and is widely associated with poor neurodevelopmental outcomes. [8-12] Leitner et al. found significantly lower neurodevelopmental outcomes in IUGR children with 28.6% having suboptimal neurological outcomes. [13] Additionally, a systematic review done by Murray et al. not only found that IUGR increased the risk of neurodevelopmental impairments, but those displaying evidence of circulatory redistribution during gestation, an indicator of asymmetric IUGR secondary to placental insufficiency (PI), were more severely affected. [14] IUGR is also associated with increased risk of developing cerebral palsy (CP), autism spectrum disorder, and attention-deficit hyperactivity disorder. [9,15,16]

Antepartum insults to the developing brain result in different pathophysiology and severity, correlating to the time and duration of injury. [17] Insults in early gestation may result in abnormal proliferation and migration, with inappropriate cell localization, whereas injury near the end of gestation may lead to abnormal differentiation and gliogenesis. Injury at any developmental stage can result in NDD. In this regard, Back et al. determined that the window of vulnerability for periventricular leukomalacia coincided with oligodendrocyte (OL) maturation, specifically 23-32 weeks gestation. [18] Interestingly, Strickland hypothesized that the same type of insult causes CP, autism spectrum disorder, and attention-deficit hyperactivity disorder, but the timing of injury consequently determines which NDD establishes. [19] It was further hypothesized that the use of a dietary supplement with antioxidant properties could potentially prevent these disorders. [19]

1.1.1 Cerebral Palsy

IUGR offspring have been shown to demonstrate long-term behavioural, cognitive, and motor abnormalities reminiscent of a CP phenotype [manuscript in progress].[20] CP is a well-recognized NDD, defined by Rosenbaum et al. as a group of non-progressive motor impairments "*often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour...*".[21] The incidence of CP is 2-2.5/1000 term births and increases ten fold in pre-term births making it the most common motor disorder in childhood.[7,22] Despite medical advancements the rate of CP has not declined.[23] Moreover, a Cochrane review by Stock et al. showed that early delivery of compromised fetuses does not improve outcome and more children had CP at two years old.[24] CP affects males at a higher rate than females (1.4:1), and the lifetime economic burden of CP per individual is an estimated 1.5 million US dollars.[25,26] The etiology of this debilitating disorder is heterogeneous and multifactorial. Several risk factors include inflammation, congenital defects, anoxia, trauma, low birth weight, and preterm birth.[27,28] Of these, low birth weight and prematurity present the highest risk of CP development, with decreasing gestational age and weight increasing the risk.[16] Although injury to the developing brain may occur prenatally, during birth, or postnatally, the majority of CP cases (70-90%) are due to antepartum insults.[29] IUGR is one of the most common antepartum risk factors associated with CP.[16,27,28] In fact, Blair et al. showed that IUGR correlated to a 10 to 30- fold increase in the risk of developing CP.[9] Moreover, McIntyre et al. showed that IUGR contributed to CP substantially more than birth asphyxia and inflammation.[30]

The diverse brain abnormalities associated with CP include periventricular leukomalacia, diffuse gray matter injury, and cerebral vascular accident.[31] Periventricular leukomalacia, a

white matter injury highly associated with CP, is of highest risk during 23-32 weeks of gestation, when preOLs dominate the ventricular region of the brain. [18,32-34] preOLs are particularly vulnerable to oxidative stress (OS) and inflammation. Injury to these cells can result in cell death, impeded OL maturation, and ultimately lead to periventricular leukomalacia. [32,33,35,36] The vulnerability of preOLs is due to the immature antioxidant system, immature vasculature, and the abundance of microglia in white matter. [32,35] Subplate neurons, essential for normal neural network development, have also been shown to peak around week 24 of gestation and are similarly vulnerable to hypoxia-ischemia induced OS. [37,38] The disruption of these neurons is suggested to be responsible for the gray matter abnormalities found in CP, including those in the thalamus, globus pallidus, and the cortex. [39] Interestingly however, Towsley et al. reported that approximately 32% of CP individuals displayed non-specific or normal brain scans, suggesting diffuse brain injury or some level of recovery, demonstrating the complexity of this disorder. [31]

Additionally, CP presents a wide variety of physical phenotypes. These phenotypes can be broadly divided into three major categories, spastic, which represent 85% of cases, dyskinetic, and ataxic. [40] Spastic CP is characterized by stiffness or contractions of the muscles, while dyskinetic CP contrastingly manifests as involuntary movements. Furthermore, these classifications differ in that spastic CP results from damaged pyramidal neurons and occurs more frequently in preterm infants, whereas dyskinetic and ataxic CP are a consequence of extrapyramidal neuron injury and are more common in term infants. [41] The Gross Motor Function Classification System is also used to classify CP patients into five levels based on the severity of their motor impairment. Fortunately, the majority of CP patients (~60%) have a mild motor impairment, falling into level I-II on the scale, and are comprised of a spastic hemiplegia

phenotype, which constitute those most likely to benefit from rehabilitative therapy.[42-45] CP often co-occurs with other NDD including autism.[46] Thus, the complexity and heterogeneity of this disorder is evident in the clinical presentation, motor impairment, and etiology, ensuring a different manifestation in every individual. This heterogeneity combined with the high prevalence and burden of CP, demands a preventive therapeutic intervention that targets many risk factors and an exceptional range of pathologies, which currently does not exist.

1.2 Intrauterine Growth Restriction

1.2.1 Incidence and Clinical Classification

IUGR is broadly described as suboptimal fetal growth based on a genetically determined growth potential.[47] This pathological restriction of growth is one of the leading causes of perinatal morbidity and mortality, second only to prematurity.[48-51] The incidence of IUGR is estimated to be approximately 9% in developed countries and 23.8% worldwide, affecting up to 30 million newborns per year.[47] Several factors increase the variability in incidence including population, gestational age, and race. Currently, the clinical definition utilized for both IUGR and small for gestational age is a fetal weight below the 10th percentile for gestational age and sex, and less than 2500g at term. Unfortunately, the lack of an internationally recognized clinical definition distinguishing IUGR from small for gestational age confuses constitutionally small and pathologically small (IUGR) newborns. In light of this, clinical diagnosis and management of at risk children is challenging. Alternative methods to delineate the differences between small for gestational age and IUGR have included the use of maternal characteristics and Doppler blood flow assessments alongside fetal growth curves and biometric measures.[52,53] Although, the integration of several indicators of IUGR may provide early classification, preventing

misdiagnosis and loss of valuable resources, the small for gestational age definition is still recognized as the best clinical surrogate for classifying IUGR. In Canada, between 2001-2010, the incidence of preterm and small for gestational age births, below 10th percentile, has generally stabilized at 8% of live births. [54] Although incidence has stabilized, medical advancements have increased the survival rate of these infants concurrently increasing the prevalence of associated neurodevelopmental complications. [55]

IUGR infants are classified into symmetric and asymmetric subtypes; with the majority of cases (70-80%) attributed to the latter. [47] Symmetric IUGR occurs earlier in pregnancy and displays a proportional reduction in both brain and body weight as well as length and head circumference. [47,56] Although symmetrical IUGR is less common, due to the disruption in growth early in pregnancy these infants are at greater risk of adverse outcomes and more severe neonatal complications including an increased risk of death, compared to asymmetrically restricted infants. [57] This type of IUGR can be difficult to distinguish from small for gestational age fetuses due to proportional similarities. On the other hand, late-onset asymmetrical growth restriction is characterized by 'brain sparing', and typically occurs in the third trimester of pregnancy. [47,56,58] 'Brain sparing', refers to maintaining brain and head circumference at the expense of other vital organ growth and soft tissue mass to increase the probability of survival. [58,59] Unfortunately, Duncan et al. showed that the maintenance of head circumference seen in typical diagnostic measures does not reflect the changes in brain growth occurring in asymmetrical IUGR. [60] This suggests that this phenomenon does not necessarily afford protection against neurological deficits caused by subtle alterations. In this regard, as previously mentioned, asymmetrical IUGR significantly increases the risk of neurodevelopmental impairments, NDDs, and severe cognitive

deficits. [9,14,57] Practically, symmetric and asymmetric IUGR are distinguished using the cephalization index (head circumference divided by birth weight), as asymmetric fetuses have an increased head circumference compared to body weight. [61]

1.2.2 Etiology

Fetal growth is dependent upon several factors maternal, placental, and fetal in origin. Under normal circumstances the maternal-placental-fetal units provide the support to reach an inherent growth potential resulting in a healthy newborn. Disruptions to any part of this unit may result in a failure to thrive and ultimately IUGR. Maternal factors that can lead to the development of IUGR include age of conception, socioeconomic status, substance abuse, malnutrition, and medical disorders. [47,62] Abnormal uteroplacental vasculature, placental abruption, and abnormal cord insertion, are placental factors responsible for IUGR development. [47,62] The majority of fetal causes of IUGR are genetic. [47] However, multiple gestations, congenital abnormalities such as cardiovascular defects, and infections of the fetus can also result in IUGR. [47,62] Although, IUGR can result from a combination of factors, most cases of asymmetrical IUGR are attributable to PI. [63,64]

1.2.3 Placental Insufficiency

The role of the placenta is to transfer oxygen and nutrients from maternal to fetal circulation. The transfer of these substrates is essential for normal fetal growth and development. Following implantation of the blastocyst, trophoblast cells differentiate into the inner cytotrophoblast layer and the outer syncytiotrophoblast layer. [65,66] The deep syncytiotrophoblast

layer forms vacuoles that upon further invasion fill with maternal blood, establishing uteroplacental circulation. [65,66] Cytotrophoblast cells penetrate the syncytiotrophoblast layer to form a complex system of fetal villi. [65,66] These villi undergo vasculogenesis forming fetoplacental blood vessels connected to the embryo by a primitive umbilical cord. [65,66] Moreover, cytotrophoblast cells erode the maternal spiral arteries, filling the intervillous space with maternal blood, to form the primary site of gas and nutrient exchange between mother and fetus. [65,66] As the fetus undergoes rapid growth throughout pregnancy, metabolic demands are met by an increased blood flow, increased surface area to volume ratio of villi, and growth of the placenta. [65,66]

PI describes any dysfunction of the placenta resulting in the insufficient supply of oxygen and nutrients to the fetus. Placental dysfunction can arise from poor trophoblast invasion, abnormal villi development, placental abruption, and thrombosis. [66] The insufficient blood flow caused by these alterations can lead to chronic fetal ischemia and IUGR. A compromised placenta is particularly detrimental during the last trimester of pregnancy due to a surge in fetal growth. The inability to meet the fetal demand for oxygen and nutrients during this critical period can lead to the redistribution of blood from the body to the brain causing the 'brain sparing phenomenon'.

1.2.4 Animal Models of IUGR

Experimental models of IUGR generally utilize maternal malnutrition, maternal hypoxia, or disruptions to placental function or blood flow. [67] Maternal malnutrition is the most common cause of IUGR in under-developed countries, while maternal hypoxia mimics high altitude

pregnancies and conditions of maternal smoking.[47] Maternal malnutrition and hypoxia models only explore each component crucial to fetal growth (oxygen and nutrient supply) in isolation. In fact, Johnston et al. suggests that injury to the developing brain is caused by the combination of hypoxia and ischemia rather than hypoxia alone.[68] As mentioned above PI reduces both oxygen and nutrient supply to the developing fetus, encompassing both components key to prenatal growth. PI has been explored through alterations of either placental function or blood flow in various species.[67]

Derrick et al. developed a model of PI in pregnant rabbits that temporarily occluded the descending aorta.[69] Offspring of this model display white matter damage and developmental delay, however the severity of injury demanded intensive care, decreased offspring viability, and reduced the feasibility of long-term assessments.[69] Rosati et al. investigated IUGR by electrically inducing placental injury in pregnant rabbits.[70] Although IUGR with brain sparing was seen, the viability of these offspring was not assessed, dosage and timing of injury during gestation resulted in varying levels of growth restriction, and this model poses similar disadvantages as mentioned previously.[70] Umbilical artery occlusion has also been studied in sheep to determine the physiological interactions of the maternal-placental-fetal unit.[67] However as precocial animals, mimicking preterm injury is difficult in sheep and long-term studies are impractical due to expensive housing costs and long gestational periods.

Although all animal models have value, rats are extremely hardy, cost effective, can be utilized to evaluate long-term outcomes, have short gestations with multiple offspring, and their brain development has been extensively characterized and compared to humans.[71] Although rat

brains are lissencephalic and development is considerably more rapid, it encompasses the same maturational milestones as human development. [71] Neurogenesis begins at the start of the second week of gestation in rats, roughly reaching completion by the fifteenth day of life. [71] Human neurogenesis also predominately occurs during gestation. [71] Additionally, gliogenesis begins during gestation and continues into the postnatal period in both rats and humans. [71] Through the evaluation of brain volume Dobbing and Sands concluded that seven-day-old rat brain development was equivalent to a term human infant, and a three-week-old rat brain corresponded to a 2-year-old human. [72] Moreover, the second half of rat gestation was found to resemble the second trimester of human brain development. [72] This resulted in the utilization of term and week old pups to investigate human antepartum and perinatal injury, respectively.

Moreover, rats have similar placentation to humans including gross morphological shape and cell layers, making them a suitable model to investigate placental dysfunction. [73] Unilateral and bilateral uterine artery ligation in rats has been well established as a model of PI induced IUGR. Wigglesworth developed the unilateral uterine artery ligation model in 1964, and it has since been widely applied. [74,75] However, due to the anatomy of a rat uterus unilateral ligation may cause contamination in the ligated horn by providing collateral blood flow from the contralateral uterine artery, interfering with the intended insult. Consequently, classification of pups based on weight following natural delivery in this model is flawed. Normal sized animals may survive from the ligated horn due to positioning nearest the ovarian artery and low weight animals may be runts from the non-ligated horn. Therefore, the utilization of bilateral uterine artery ligation, which circumvents these issues, provides an exceptional model of PI induced IUGR. Moreover, the hallmark features of asymmetric IUGR are mimicked in this rodent model

suggesting that results may be extrapolated to the human condition, although there are inherent limitations when translating findings from animal studies to human conditions. [76,77] Previous work in our laboratory utilized this model in both short term and long term studies verifying validity and demonstrating its reproducibility [manuscript in progress]. [20,76] Furthermore, the use of Long-Evans rats can provide the opportunity for long-term studies evaluating therapeutic interventions, due to their robustness for behavioural testing. [78]

1.3 Oxidative Stress

Although largely unknown, the underlying cellular mechanisms of abnormal brain development in asymmetrical IUGR have been associated with fetal OS. [79] Karowicz-Bilinska et al. found increased indices of OS, increased lipid peroxidation measures, and decreased total antioxidant capacity in the blood serum of pregnant women diagnosed with IUGR fetuses, secondary to PI. [79] Markers of lipid peroxidation were also detected in the umbilical vein of IUGR newborns as compared to their normally developing twin. [80] OS arises when the balance of oxidants and antioxidants is altered by the accumulation of free radicals. Free radicals are highly reactive chemicals with one or more unpaired electrons in the outer orbital. These free radicals are derived from molecular oxygen and nitrogen, existing as reactive oxygen-nitrogen species (ROS). Due to the unstable state caused by unpaired electrons, ROS react with surrounding molecules to stabilize. The altered equilibrium in favour of free radical production has huge implications for the developing brain due to its vulnerability to oxidation.

OS is attributed to the ischemic state of PI. During ischemia, reduced oxygen supply causes a switch in adenosine tri-phosphate (ATP) production from oxidative phosphorylation to

the inefficient anaerobic glycolysis. [81,82] Lactic acidosis caused by the conversion of glucose to lactate is one of the sources of ROS production. [82,83] In this condition glucose availability is also reduced, expediting the exhaustion of brain glucose stores and subsequent energy failure. This energy failure results in the influx of intracellular calcium (Ca^{2+}), causing further amplification of ROS production. [82,84-86] Moreover, during ischemia, the reduced oxygen availability disrupts the electron transport train of ATP synthesis, generating superoxide in the mitochondria. [87] These volatile ROS selectively target phospholipid membranes, proteins, and nucleic acids, disrupting the normal structure and function of cells. [88] Additionally, OS can compromise fetoplacental blood flow, further potentiating the ischemic state and creating a vicious cycle that compromises fetal growth and development. [66,89]

The fetal brain is highly susceptible to OS due to an immature antioxidant system, high rate of energy consumption, rich amount of unsaturated fatty acids, and an abundance of redox-active iron and copper. [32,90-92] The impact of OS on astrocytes, microglia, and neurons, for the purpose of this study, is illustrated in Figure 1. Due to the lack of histones and few repair mechanisms a predominant target of OS is mitochondria, the site of energy production in the cell. [93] ROS attack mitochondrial DNA resulting in mitochondrial dysfunction, energy failure, amplification of ROS production, and eventually cell death. [93] The abundance of polyunsaturated fatty acids is responsible for the high metabolic rate and energy consumption of the brain. [90,94] Unfortunately, polyunsaturated fatty acids, which are particularly abundant in neuronal membranes, are prone to ROS attack, resulting in lipid peroxidation, decreased membrane potential, and subsequent membrane rupture. [88,90] The high levels of iron in the brain can further promote OS by catalyzing ROS production and lipid peroxidation.

Unless the chain reaction of excessive ROS production is broken by antioxidants, chronic microglial activation can occur. [94] Microglia function as brain-specific macrophages and once activated, provide neuronal support by determining if a neuron can be saved or cleared. [94] Microglial activation can lead to a positive feedback loop of microglial dysfunction, inducible nitric oxide synthase activation, and cytokine release, resulting in a neurotoxic state. [94] In this toxic state neurons undergo axonal and dendritic retraction, reduced spine density, and neurotransmitter (NT) release, culminating in death. Additionally, immature neurons have a greater propensity for apoptosis than mature neurons due to natural elimination processes responsible for forming optimal neural networks. [95,96] These impairments in neuronal structure and function can result in cognitive and behavioural abnormalities. Furthermore, OS targets immature preOLs, decreasing the number of mature OLs, which leads to additional functional abnormalities. [97] Ultimately, the combined effects of energy failure, intracellular Ca^{2+} accumulation, excitotoxicity, and ROS production due to ischemia cause deleterious effects on the cell. Therefore, maintaining the redox balance between oxidants and antioxidants is crucial for brain cell survival, particularly the vulnerable neurons.

Although, ROS serve as signaling molecules for the induction of cell differentiation and proliferation during fetal development, overproduction can overwhelm the antioxidant supply. [98] Preservation of the delicate redox balance occurs through the utilization of endogenous antioxidants such as glutathione (GSH) and exogenous molecules like vitamin C and polyphenols. GSH is the most abundant endogenous antioxidant in the cell and is recognized as the first line of defense against ROS. [99,100] The synthesis of the tripeptide GSH requires glutamate, cysteine (Cys), and glycine to combine in two ATP-consuming reactions that are

catalyzed by glutamate-cysteine ligase and glutathione synthetase (Figure 2). [100] GSH not only serves as an efficient electron donor directly detoxifying ROS, but also reacts with ROS in a recycling pathway (Figure 2). [30,31] In the recycling pathway, GSH is converted to oxidized GSH when in contact with ROS. After oxidation, catalyzed by glutathione peroxidase, oxidized GSH can be reduced back into GSH by glutathione reductase, completing a cyclic reaction optimal for combating OS. [100,101] In contrast, GSH is directly conjugated to ROS by glutathione-S-transferase, effectively lowering cellular GSH. This sophisticated antioxidant defense system also includes efficient enzymes such as superoxide dismutase and catalase. Superoxide dismutase breaks down the ROS superoxide into oxygen and hydrogen peroxide, while catalase and glutathione peroxidase further breakdown hydrogen peroxide before it can be converted into hydroxyl radicals by metal ions (Figure 2). [88] Unfortunately, catalase and glutathione peroxidase only reach adult levels in white matter around 30 weeks gestation, while superoxide dismutase does not fully develop until term, hindering detoxification abilities and increasing OS vulnerability. [102]

Astrocytes, another type of glial cell, can protect neurons from oxidative damage through their antioxidant systems. [103] Astrocytes actively combat OS through the nuclear factor E2-related factor 2 (Nrf2) pathway, indirectly supporting GSH metabolism and release, as well as the release of ascorbate. [104-108] Tanaka et al. demonstrated that astrocytes increase neuron survival under OS in vitro, which was assumedly due to the antioxidant capacity of astrocytes. [109] Notably, astrocytes are the predominant source of GSH in the brain. [108,110-112] Astrocytes supply essential GSH precursors to neurons, which allows for the maintenance or increase in neural GSH, as demonstrated in co-cultures. [112,113] On the other hand, astrocyte antioxidant systems can

become overwhelmed resulting in reactive astrogliosis and contributing to the underlying pathological condition. [114] The reactivity of astrocytes is on a spectrum. [115] Mild to moderate astrogliosis is generally associated with little changes to the structure or function of astrocytes and has the potential for resolution if the trigger is resolved. [115] Severe reactive astrogliosis results in structural changes and functional failure of the astrocyte, cytokine and ROS production, decreased NT uptake, Ca^{2+} influx, and excitotoxicity through glutamate release. [115-118] The ability of astrocytes to both protect neurons from OS and perpetuate cytotoxicity demonstrates the importance of neural-glia interactions and their potential as therapeutic targets.

1.4 Fetal Brain Development

PI induced asymmetrical IUGR occurs during the third trimester of pregnancy, a period of rapid growth and development of the fetal brain. During this time period neurons are migrating and differentiating, synapses are being formed, and gliogenesis is occurring. [119,120] Therefore, the timing of this insult may disrupt neuron maturation and synaptogenesis explaining developmental difficulties associated with IUGR. To this end, IUGR has been shown to alter brain volume, connectivity patterns, gray matter, and myelination. [121-127] Interestingly, Padilla et al. showed that IUGR preferentially affects gray matter over white matter, suggesting differential vulnerability. [125] In this section neurogenesis, synaptogenesis, and gliogenesis will be discussed to understand cell vulnerability during development and how antepartum disruptions can result in neurodevelopmental complications.

1.4.1 Neurogenesis

The production of the billions of neurons that make up the human brain is essentially complete by mid-gestation. To accomplish this task, the pool of neural progenitor cells must first proliferate in the ventricular zone through multiple rounds of mitotic cell division. [119] Following this rapid increase in the progenitor cell population, asymmetrical cell division takes over, producing one neuron and one neural progenitor. [119] The next step of neurogenesis is the migration of the post-mitotic neurons from the ventricular zone throughout the brain. In the cortex, migration begins with the first neurons forming the preplate, which will split into the marginal zone and subplate with successive migration. [119,128] These transient layers allow the formation of the cortical plate comprised of the six layers of the cortex. The six cortical layers are composed of different types of neurons that stem from the same neural progenitor cells depending on environmental cues. [129] Migration to the deepest of the six layers by the earliest neurons is completed through somal translocation, the extension of a basal process to the outer surface. [128] Subsequent migration utilizes radial glial guides that form a scaffold to the outer surface. [119,128,130] Neurons attach to this scaffold and are guided through the cortical plate to the marginal zone. [119,130] The marginal zone contains Reelin signaling Cajal-Retzius cells that stop the migration of the neuron. [119]

Maturation integrates the neurons of varying cortical and subcortical regions into a complex neural network. The ability of the neural network to communicate and process information begins with the development of neuronal processes known as axons and dendrites. Generally, the axon, a single long projection that branches extensively to establish connections with target cells, sends signals, while the dendrites receive and integrate input from surrounding

neurons. [119] The diversity of neural structures in the mammalian brain is extraordinary and classified by their function or morphology, which depends on their location in the central nervous system. [119,120]

Proper nervous system functioning hinges on the correct growth and arborization of the dendrites. The patterns, complexity, and length of dendritic arbors determine the innervation received by a neuron and ultimately define its communicative and processing capabilities. [131] Moreover, proper dendritic development dictates the number and pattern of synapses formed. [131] The correct wiring of the neural network relies on the navigation of dendrites to their target locations using specialized growth cones. [132,133] Transient dendritic protrusions known as filopodia rapidly extend toward growth cones and guidance cues increasing arborization and synaptic spine formation. [132-134] Arborization of dendrites can occur by branching via bifurcation of the filopodia or interstitial sprouting from existing branches. [134]

The dynamic growth of dendrites utilizes both intra and extracellular guidance cues. [131] Roger Sperry's hypothesis of chemoaffinity postulated that each growing axon has a unique molecular identity that either attracts or repels filopodia, directing proper dendritic growth. [135,136] This theory has since been validated across various organisms with the discovery of cues such as semaphorins and ephrins. [132,137] Dendritic growth is also regulated by specific patterns of synaptic activity and neurotrophic factors. Neurotrophic factors, including brain derived neurotrophic factor and neurotrophin 3 preferentially effect active neurons by binding to tyrosine kinase and p75 receptors. [138] These receptors are abundant in the fetal brain during neuronal growth and differentiation, and differentially influence the response of a neuron to neurotrophic

factors based on the unique combinations that they express. [138,139] The activity of neurotrophins can change dendritic patterns by either enhancing growth or retraction. McAllister et al. showed that different neurotrophins can have opposing effects within a cell type, as dendritic growth in layer 4 cortical neurons was stimulated or inhibited, based on the administration of brain derived neurotrophic factor or neurotrophin 3 respectively. [140] Moreover neurotrophins can differentially affect different cell populations, for example, brain derived neurotrophic factor can stimulate dendritic growth in layer 4 cortical neurons while inhibiting growth in layer 6. [140]

The prevention of dendritic overlap and redundant innervation of functionally similar neurons occurs through 'tiling'. [139] In tiling, dendrites of the same subtype repel each other in a like-repels-like manner preventing overlap between and within neurons. [141] The suggested underlying mechanisms of tiling include interactions through contact and short range signaling. [141,142] Once the final target is established filopodia become more static, decline in number, and synaptogenesis occurs. [134,143] Additionally, in the first two years of life over half of the connections made during development are eliminated. [144] Exuberant dendritic connections are pruned through neurite elimination, which sculpts the mature neural network. [145] Exuberant connections prevent abnormalities in connectivity by allowing initial mistakes to be corrected through selective elimination. Under normal conditions connections with low synaptic innervation undergo caspase dependent fragmentation. [145,146]

Cytokines, chemokines, NTs, and neurotrophic factors that guide and influence neuronal growth have been implicated in neurological complications. [147-152] This illustrates how insults during neurogenesis can subsequently alter the delicate conditions required for normal neural

development. IUGR may disrupt these balances, due to OS, altering neurite growth and arborization. In studies by van Vliet et al. and Simoes et al., IUGR rabbit kits displayed reduced metabolites responsible for neuron and OL viability, density, structure, and energy metabolism. [153,154] Moreover, Hernandex-Andrade et al. showed alterations in glutamate and dopamine levels of IUGR rabbit offspring in the cortex, striatum, and hippocampus. [155] Due to the importance of neuronal morphology in proper neural network communication and formation, it is reasonable to conclude that disruptions during neuron maturation can result in NDD.

1.4.2 Synaptogenesis

The billions of neurons within the neural network communicate via synapses, spaces between neurons. Synapses permit communication through action potential induced chemical diffusion. In the mammalian brain, postsynaptic dendritic spines, specialized protrusions, receive the majority of these signals and are therefore important for experience dependent plasticity, learning, and memory. [156-158] The evolution of these 0.5-2μm long protrusions indicates their necessity in an advanced and complex nervous system as they are rarely found in lower organisms. [159,160] Spines emerge from the dendritic shaft to make synaptic connections with postsynaptic terminals early in postnatal life. [71] Spines are also formed by dendritic filopodia, particularly during early synaptogenesis. [161,162] As previously mentioned, dendritic filopodia are highly motile and repeatedly form transient contacts with axons. Once these contacts are made, filopodia undergo morphological and functional transformations into spines. [161,162]

Immature spines with long necks and small heads form rapidly and at random. Studies using time-lapsed fluorescent imaging show the rapid formation and morphological alterations of

spines, which can occur throughout life. [163-165] The dynamic morphological alterations of excitatory dendritic spines provide astonishing plasticity. The maturation of dendritic spines leads to an increase in size and morphological changes of the head. The head of the spine directly apposed the active synapse can thicken into what is known as the postsynaptic density. [160,166] The postsynaptic density contains NT receptors and signaling proteins consequently specializing its ability for postsynaptic signaling. [166] The postsynaptic density also contains adhesion molecules that bind pre and postsynaptic cells together for stability. [166] The presynaptic terminal and postsynaptic spine are separated by a 20-25nm gap, the synapse, where neurotransmission occurs. [166] In addition to containing the postsynaptic density, the size of the spine head correlates to the size of the synapse, number of postsynaptic receptors, presynaptic vesicles, and diversity of organelles it contains. [160] These factors suggest that the stabilization and maturation of the spine head relates to the strength of synaptic activity, connectivity, and ultimately neural function.

The density of dendritic spines correlates to environmental stimulation and is associated with developmental disability. [160,167] For example, decreased spine density has been shown in the prefrontal cortex of schizophrenia patients. [168] The dynamic abilities of spines allow neural compensation to changes in synaptic activity. Overall spine density decreases with overstimulation, and increases with insufficient stimulation, allowing the maintenance of excitatory homeostasis. [160] As dendritic arborization, spine development, and synaptic transmission are directly related to receiving and integrating information it is not surprising that abnormalities can lead to improper brain development. [169]

1.4.3 Pyramidal Neurons

Pyramidal neurons (PNs) are found in structures associated with higher cognitive functioning such as the cerebral cortex and hippocampus. [170] In addition to being the most abundant type of neuron in the cortex, the axons of PNs project to other cortical and subcortical regions. [171] PNs are unique due to the characteristic separation of the dendritic tree into basal and apical domains. The several short basal dendrites and long thick apical dendrite emerge from the base and apex of the triangular shaped cell body, respectively. Typically, there is one main apical dendrite, with extensive oblique branching, that bifurcates into a terminal tuft at the distal end. The bi-conical anatomical separation and the distinct morphological differences of the PN domains suggest functional significance. This segregation allows the reception of information from different afferent sources, which are then separately integrated. [170,171] PNs therefore have the potential to operate multiple integration functions or learning rules simultaneously, and changes to their structure can alter these sophisticated processes. [171]

In the cortex basal dendrites occupy the same layer as the cell body and generally receive feedforward information while the apical dendrites ascend to superficial layers and provide feedback information. [171] Similarly, in the CA1 region of the hippocampus basal dendrites receive information from neighbouring CA1 and CA3 neurons while apical dendrites integrate input from the entorhinal cortex. [170] The inputs received in the separate domains differentially impact action potential (AP) production due to their differences in size, geometry, and electrical conductance. [171] Activation of apical dendrites has a weaker effect on AP production due to electrical impedance, unreliable propagation, and smaller, prolonged excitatory postsynaptic potentials. [171-174] This evidence suggests that apical activation has a modulatory role over the

basal inputs that primarily drive AP production. [171-173] Input processing is further complicated by the possibility of behavioural conditions impacting the mechanisms of integration. For example, dendrites may respond as coincident detectors in some situations, while apical modulation of basal inputs may dominate in others. [170,171,175,176]

APs are initiated at the axon initial segment after integration of all dendritic activation. Voltage-gated channels, which promote AP propagation, impact the AP firing of pyramidal neurons by influencing intrinsic AP threshold, hyperpolarization, and depolarization. [177] Along with dendritic morphology, the variability in these channels increase the diversity of PN function and impacts the firing pattern of the cell. [177] Although the underlying mechanisms are unknown PNs can exhibit tonic-spiking intervals or burst firing, clusters of APs with short interspike intervals, with varying spike frequency. [178] Burst firing is crucial to neuronal signaling and synaptic plasticity as they are more reliably transmitted, induce long-term potentiation, convey specific stimulus input, and improve the signal-to-noise ratio of responses. [179-182] Bursts are generated in what was termed a 'ping-pong' interaction by Wang in 1999. [183] After the generation of an AP, hyperpolarization of the cell body causes a return current and the depolarization of dendrites, mediated by voltage-gated channels. [178,183] This depolarization, if strong enough, can elicit another AP, thus the occurrence of burst firing.

The morphology of the dendrites plays a critical role in the development of burst firing. [178] van Elburg and van Ooyen, found that apical dendritic length (DL), topology, and symmetry influenced neuronal firing patterns, the number of spikes in each burst, and the interspike intervals in a computational model of cortical pyramidal cells. [178] A reduction of total DL

changed the firing pattern from burst to tonic, suggesting a functional alteration of the neuron. [178] They also found that ion channel densities did not affect the changes elicited by apical morphology, suggesting that the loss of burst firing as a result of dendritic tree reduction cannot be saved by compensatory channel changes. [178]

1.4.4 Gliogenesis

Neural stem cells begin to differentiate into glial progenitor cells following neurogenesis, in the late embryonic and early postnatal period. [71,119] Several intrinsic and extrinsic factors are involved in the temporal switch of stem cell differentiation from neural progenitor cells to glial progenitor cells including the Janus kinase/signal transducers and activators of transcription pathway, bone morphogenetic protein, notch signaling, and activation of proglial genes. [184] Glial progenitor cells differentiate into OLs and astrocytes after migration throughout the brain. Astrocytes are traditionally known as resident neural support cells and are involved in many essential functions such as ion and transmitter homeostasis, synaptic transmission and formation, blood-brain barrier development, blood vessel integrity, and the release of trophic factors. [185] Identification of these cells is based on their irregular star-like cell body. Immature astrocytes migrate throughout the central nervous system before expressing glial fibrillary acidic protein, maturing into ramified cells, and contacting blood vessels and neurons with 'endfeet'. [185,186]

Following astrocyte production, OL progenitor cells begin to differentiate. As OLs mature the structures become more complex, expressing unique combinations of markers at each stage. [33] OL progenitors give rise to preOLs that proliferate and migrate throughout the brain before developing into myelin producing mature OLs. [33,187] The main function of an OL is the

myelination of axons, which provides insulation that increases the conduction velocity of neuronal APs. [187] OLs have also been shown to synthesize trophic factors aiding in axonal integrity and neuronal survival. [187] The laying down and correct spacing of myelin along the axon is coordinated by the communication between neurons and OLs. [188,189]

1.4.5 Neuron-Glial Communication

Intricate neuron-glial signaling, although not fully understood, demonstrates the complexity of normal nervous system functioning. Glial cells contain many ion channels and NT receptors that allow the indirect monitoring of neuronal activity by chemical changes in the environment. [190] These chemical signals are the primary basis of neuron-glial and glial-glial communications. [188] The bi-directional interaction between neurons and glia can occur at both synaptic and nonsynaptic regions. Further evidence for neuron-glial interactions is the coordination between gliogenesis and the rapid growth of dendrites and synapses. [71,191] In fact, synapses rapidly form only after astrocyte development. [191-193]

The extensions of astrocytes are intimately associated with the synapse and have been shown to integrate, process, and regulate synaptic transmissions. For example, the release of a variety of substances during neuronal activation can regulate astrocyte excitation, evidenced by changes in cytoplasmic Ca^{2+} concentrations. [194-196] This astrocytic activation can regulate the strength of adjacent synapses by differentially affecting NT release or uptake and activation of inhibitory neurons. [188,197] Interactions between neurons and astrocytes are also thought to be involved in energy production. [198,199] Astrocytes can also communicate between each other over long distances, by the release of ATP or glutamate, or close range through contact-mediated

signaling. [188] The release of ATP can lead to a wave of Ca^{2+} changes that spread via gap junctions through astrocyte circuits. [190,194] The ability to communicate long distances together with the clearing of NTs and regulation of the extracellular environment allow astrocytes to process information and regulate synaptic strength and efficacy. [188] The interactions between neurons and astrocytes have also been referred to as a 'tripartite synapse', as the astrocyte has a functional role in synaptic physiology. [200,201]

Neural activity, growth factors, and special axon-glial signaling molecules mediate neuron-OL interactions. [188] Depending on developmental stage and location, neural activity can initiate myelination or inhibit proliferation of OL through activity-induced axonal secretions. [188] Additionally, the presence of OL adhesion molecules can differentially affect neuron outgrowth depending on the developmental stage, i.e. enabling outgrowth of embryonic neurons but blocking outgrowth postnatally. [202] Moreover, neuronal-OL communication is essential for the formation and maintenance of the nodes of Ranvier, repeating amplifiers along the axon. Yin et al. found that miscommunication between neurons and OLs by the loss of adhesion molecules leads to the degeneration of axons. [203] Furthermore, when OLs are damaged or lost, such as in multiple sclerosis, abnormal AP conduction leads to axonal degeneration. [204] The resulting deficits from abnormal neuron-glial communications demonstrate their necessity for normal central nervous system function.

As described above, the neuron-glial interactions are essential for normal AP conduction, synaptic transmission, and information processing. [188] Disruption or damage to any glial cell in this system can therefore lead to widespread neuronal degeneration ultimately resulting in

functional changes of the nervous system. Acknowledging these cells as a system will allow further understanding of the vast computational abilities and adaptability of the brain to injury permitting the introduction of sophisticated treatments.

1.5 Current Therapies

Medical advancements ensuring the survival of preterm and compromised infants increases the risk for developing NDD, including CP. Preventing the antepartum insults that result in abnormal brain development could subsequently reduce the incidence of NDD. Although many therapies have been investigated, the use of preventive interventions has been unsuccessful in clinical trials. Unfortunately there are several factors that complicate the transition of interventions into clinical trials including timing and difficulty in treating the fetal brain. Due to the constant changes in development the brain becomes a 'moving target' and the onset and duration of injury can be difficult to elucidate making intervention application challenging. Furthermore, as safe and effective treatments for the fetal brain have not been determined, the administration of drugs during pregnancy raises ethical concerns. Delivery of these drugs to the fetus also poses a problem, as the placenta and fetal blood brain barrier may prevent distribution. Moreover, the use of neuroprotective drugs, shown to be effective in adults, is harmful to the developing brain. [205,206] The lack of current therapies to target *in utero* insults increases the demand for preventive interventions, as most postnatal treatment do not diminish long-term morbidity from altered fetal environments.

The use of drugs or dietary supplements with antioxidant properties constitutes the majority of all attempts to prevent IUGR or resulting morbidities. [76,207-211] Resveratrol, a

polyphenol found in various plants, is one such supplement that has shown beneficial results in several organ systems of IUGR offspring. These benefits are attributed to the antioxidant and anti-inflammatory properties of resveratrol. Shah et al., found that postnatal resveratrol supplementation to offspring of hypoxic rat dams reversed cardiovascular dysfunction programmed *in utero*.[212] Similarly, metabolic function was improved and cardiac OS attenuated with postnatal resveratrol supplementation in IUGR offspring of hypoxic rat dams.[213,214] Poudel et al. demonstrated the ability of resveratrol to increase uterine artery blood flow and fetal weight in catechol-O-methyl transferase knockout mice, a model of IUGR and preeclampsia.[215] In addition, Shen et al. demonstrated that resveratrol could enhance *in vitro* neural stem cell survival and proliferation when administered before oxygen-glucose deprivation.[216] Bourque et al. also showed that resveratrol can cross the placenta and improve fetal outcomes associated with maternal hypoxia in rats.[217] Despite these promising results they are not always reproducible, possibly due to variations in timing, duration, and dosage of resveratrol. Moreover, oral resveratrol is rapidly eliminated and has less biologic activity than the cis-form typically used experimentally.[218] Resveratrol has also been shown to have pro-oxidant properties depending on the concentration and cell type, which can lead to OS.[218]

1.5.1 Broccoli Sprouts

The consumption of a healthy diet including cruciferous vegetables, such as broccoli sprouts (BrSp) is a possible neuropreventive intervention. Unlike RSV, which is only a polyphenol, cruciferous vegetables contain many molecules, which have significant health benefits. Moreover, because BrSp are a natural health product there are no recognized detrimental effects of consumption based on dose.[219] Cruciferous vegetables contain

phytochemicals, polyphenols, vitamins (A, B, C, E, and K), minerals (iron and magnesium), omega-3 fatty acids, fiber, and Cys. [220] The compounds that are hypothesized to be of greatest benefit are the phytochemicals called glucosinolates. The cruciferous vegetables with the highest concentration of the glucosinolate, glucoraphanin are BrSp. [221] When glucosinolates come in contact with their enzyme myrosinase, such as in mechanical breakdown, they are converted into isothiocyanates. Isothiocyanates have been associated with preventing and reducing the risk of cancer and cardiovascular disease, as well as reducing infarct volume in stroke. [222-224] Protection is afforded by isothiocyanates due to their ability to promote endogenous antioxidant and anti-inflammatory enzyme production, thereby boosting the intrinsic antioxidant response of the cell. [225]

BrSp dietary supplementation has been shown to elicit an anti-oxidant and anti-inflammatory response. [226] These beneficial properties improve the outcomes in several experimental studies. Noyan-Ashraf et al. demonstrated the benefits of BrSp consumption not only for first generation hypertensive stroke-prone rats, but also for their offspring. [226] It was shown that first generation females, that consumed BrSp, had reduced blood pressure as well as decreased brain and kidney inducible nitric oxide synthase. [226] Moreover, the redox balance was improved as shown by an increased glutathione peroxidase, glutathione reductase, and GSH, with decreased oxidized GSH in the heart and kidney. [226] Remarkably, these results were evident in the offspring of these females, demonstrating the benefits of BrSp supplementation on the fetus. [226] Similarly, Wu et al., found that the consumption of BrSp increased GSH, glutathione peroxidase and glutathione reductase, while decreasing oxidized GSH and activated macrophages, in male hypertensive stroke-prone rats. [227]

The benefits of BrSp consumption, as a neuropreventive therapy, have also been demonstrated. Black et al. evaluated pathology, neurobehavioural development, and maturation in PI induced IUGR rat offspring with and without maternal BrSp consumption.[76] It was found that BrSp supplementation during gestation and lactation afforded improvements in newborn reflexes, open field measures, myelination, ventricular dilation, astrocyte reactivity, and hippocampal cell counts.[76] Nguyen et al. showed that maternal BrSp consumption prevented growth restriction, developmental delay, and anxiety in lipopolysaccharide induced fetal inflammation in Long-Evans rats.[228] These studies show the potential for neuroprevention of several fetal determinants of adult health using a dietary supplement, BrSp. Thus the amelioration of disorders caused by OS and inflammation may result via consumption of a healthy diet targeting the promotion of endogenous antioxidant production. The advantages to using BrSp, is that, identification of the at risk population is not required, there are no recognized adverse side effects, and the exact timing of injury does not have to be elucidated. Moreover, BrSp consumed during pregnancy was shown to provide absorption and transfer of sulforaphane (SFN) from the rat dam to the fetus, providing evidence of distribution across the placental barrier.[76]

1.5.2 Sulforaphane

As previously mentioned, BrSp are a rich source of the functional glucosinolate glucoraphanin. In fact, it has been shown that BrSp contain twice as much glucoraphanin as compared to other cruciferous vegetables and significantly more than in the mature broccoli plants.[221,229] The enzymatic conversion of glucoraphanin into the isothiocyanate SFN occurs as a result of the mechanical breakdown of BrSp. The enzyme myrosinase is naturally segregated

from glucoraphanin in a healthy plant. When released myrosinase catalyzes the hydrolysis of glucoraphanin into SFN.[225] SFN is a potent phase II enzyme inducer that boosts the antioxidant and anti-inflammatory capacity of the cell. These phase II enzymes neutralize harmful free radicals and electrophilic agents. Phase I enzymes, such as cytochrome P450 oxidase produce these electrophilic agents by introducing reactive or polar groups to xenobiotics.[230] Unfortunately, the fetal brain does not efficiently produce phase II enzymes due to the immature antioxidant defense system. Therefore, maternal consumption of BrSp during gestation may boost endogenous fetal phase II enzymes and the antioxidant defenses needed to combat OS *in utero*.

SFN facilitates the production of phase II enzymes through the Nrf2 pathway (Figure 3). Nrf2 is a transcription factor essential for the positive regulation of the antioxidant response element (ARE) found on the promoter regions of genes that transcribe detoxification and antioxidant enzymes.[231] Nrf2 induced detoxifying and antioxidant enzyme production is suppressed by Kelch-like ECH-associated protein 1 (Keap1).[232] Keap1 sequesters Nrf2 to the cytoplasm and targets it for proteosomal degradation through cullin-3 mediated ubiquitylation under normal conditions.[230,231] However, Nrf2 can be activated when the Cys residues between cullin-3 and Keap1 are disrupted by oxidative stress or SFN exposure.[230,231] The released Nrf2 is free to translocate to the nucleus, bind to the ARE along with the maf transcription factor, and initiate the transcription of endogenous phase II enzymes, including glutathione S-transferase, NAD(P)H: quinone reductase, and heme oxygenase.[225,230] The indirect antioxidant enzyme producing ability of SFN, through the enhancement of Nrf2 activation, combats oxidative stress and promotes cell survival.[230] Zhao et al. showed that SFN injections reduce infarct volume in

rats with focal ischemia by increasing levels of Nrf2-responsive heme oxygenase. [222] Moreover, SFN can stimulate the production of glutamate-cysteine ligase, which catalyzes the first rate-limiting step in GSH synthesis (Figure 2). [225] GSH is the most abundant and versatile endogenous antioxidant present in the body, which acts as the first line of defense against oxidants.

The anti-inflammatory properties of SFN provide another mechanism by which neuroprotection may occur. Holloway et al. demonstrated anti-inflammatory benefits of SFN pretreatment on lipopolysaccharide induced leukocyte recruitment and inflammatory cytokines in the mouse brain, which was dependent upon the Nrf2 pathway and NF-κB signaling. [233] Moreover, SFN pretreament induced neutrophil inhibition by the down-regulation of adhesion molecules in human brain cell lines. [233] Recently it has also been shown that SFN can inhibit inflammasomes, intracellular immune and pathogen defense complexes, independent of the Nrf2 pathway. [234] Greaney et al. showed that SFN inhibits caspase-1 activation and interleukin-1β secretion through the suppression of inflammasome activation. [234] These anti-inflammatory mechanisms by which SFN acts can further protect the developing brain, as OS can cause inflammation, and vice versa. Therefore, use of BrSp as a dietary source of SFN during pregnancy may provide neuroprevention for several NDD through the activation of Nrf2 pathway among other mechanisms.

1.6 Summary

IUGR secondary to PI is associated with NDD. This includes an increased risk of CP, autism, and attention-deficit hyperactivity disorder. [9,15,16] The cognitive and behavioural

abnormalities of these conditions may involve gray matter injury. Moreover, neurons are exceedingly susceptible to ischemic and oxidative damage. Investigating neuronal morphology may give insight into the contribution of gray matter injury in IUGR. These assessments may reveal alterations in neuronal connectivity that contributes to the risk of NDD development. Furthermore, there are no preventive therapies available to protect the vulnerable fetus from antepartum insults and subsequent NDD. BrSp may provide safe and effective neuroprevention for perinatal brain injury resulting from IUGR by targeting OS and inflammation that adversely impacts neuron development. Exploring BrSp as a novel neuropreventive intervention for abnormal neuronal morphology caused by IUGR may therefore reveal the ability to decrease adverse neurological sequellae and the subsequent risk of NDDs.

2 OBJECTIVES AND HYPOTHESIS

2.1 Overall Objective

The overall objective of this study was to evaluate the neuropreventive properties of BrSp supplementation on the morphological abnormalities of the neuron resulting from PI induced IUGR.

2.1.1 Specific Aims

To determine the effects of IUGR and maternal BrSp supplementation during pregnancy and lactation on: 1) dendritic length, 2) dendritic complexity and, 3) spine density in the CA1 region of the hippocampus and layer V of the primary motor cortex

2.2 Rationale

As indicated above, the lack of preventive therapies available to target antepartum insults, such as IUGR, significantly burdens the healthcare system. Protecting the developing brain from injury is important for the prevention of NDD. Changes in neuronal morphology, which have been shown to occur in PI induced IUGR, modify firing patterns and synaptic transmission, subsequently altering the reception and integration of information. [177,178,235] This disruption in the neural network can ultimately result in the development of NDD. Previous work in our laboratory showed developmental delays in newborn offspring, as well as behavioural abnormalities in mature rats of this model,

including spatial memory deficits, anxiety, and gross motor impairments, representative of a CP phenotype [manuscript in progress].[20,76] This previous work highlighted the hippocampus and motor regions of the brain as targets in this model. Treating the vulnerable fetal brain with a natural health product could provide a safe and effective neuropreventive therapy currently missing in clinical practice. The abundance of SFN precursors contained in BrSp mechanistically targets the Nrf2 pathway, inducing phase II and antioxidant enzymes that can theoretically combat OS caused by PI (Figure 3).[225,231] Although previous work elucidated the neuropreventive benefits of BrSp supplementation on behavioural outcomes from birth to adulthood, the observed pathology could not explain the robust behavioural effects [manuscript in progress].[20] Moreover, the literature characterizes the neuron as a target of abnormal development in IUGR offspring, which was not evaluated in these previous studies. To our knowledge, there are no studies to date that have evaluated the effect of BrSp as a neuropreventive therapy for morphological disruptions of neurons induced by PI. This study was therefore undertaken to understand how BrSp impacts neuronal morphology in IUGR offspring.

2.3 Overall Hypothesis

The primary hypothesis of this study was that, 1) PI induced IUGR will result in neurological disruptions in mature rats, and 2) BrSp supplementation would prevent these neurological abnormalities in IUGR offspring. This will demonstrate the ability of BrSp supplementation to afford neuroprevention during development that prevent the occurrence of adult disease.

2.3.1 Specific Hypotheses

Specifically, it was hypothesized that, 1) PI induced IUGR rat offspring will have altered neuronal morphology and decreased spine density in the CA1 region of the hippocampus and layer V of the hind limb primary motor cortex, and 2) BrSp supplementation will prevent these alterations from occurring.

3 INTRODUCTION FIGURES

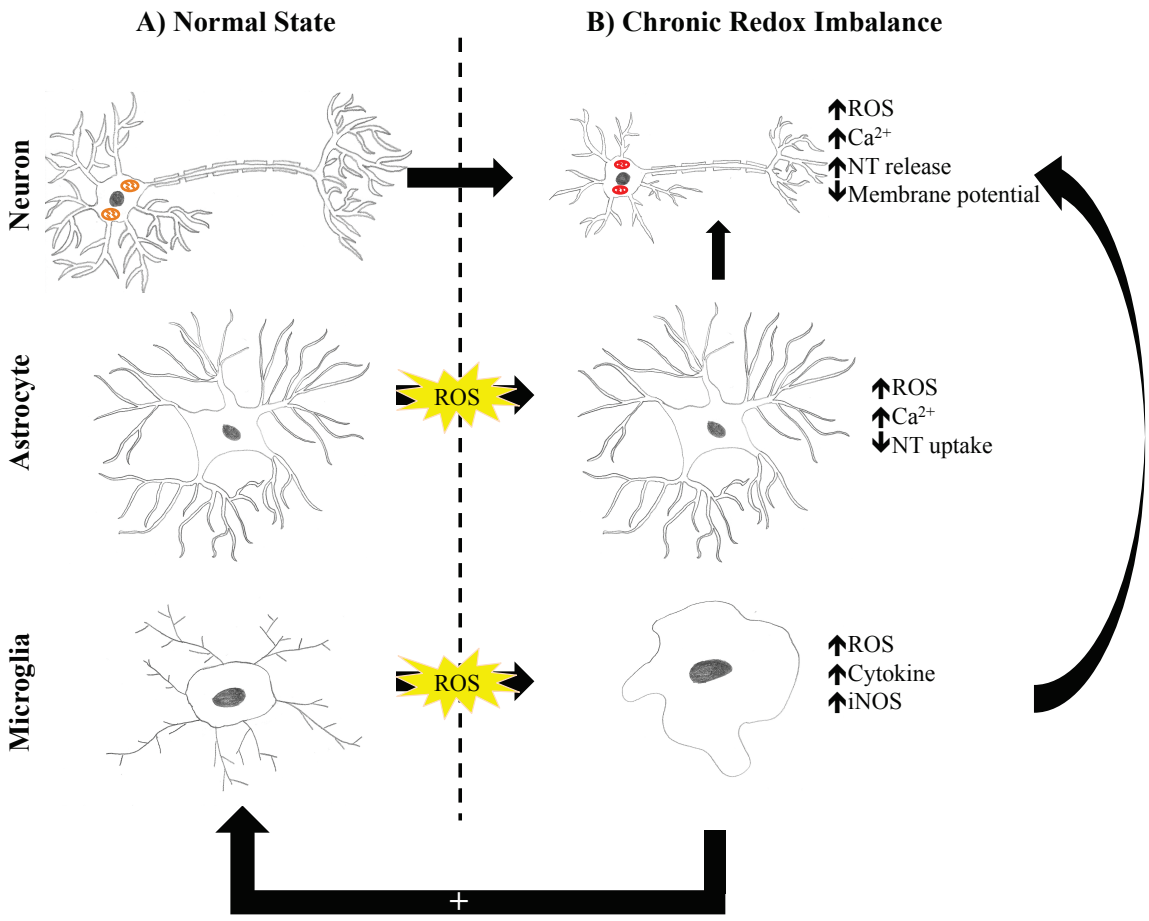

Figure 1: Impact of Oxidative Stress on Glia and Neural Development

Under normal conditions (A) dendrites are stabilized and eliminated by normal processes to refine connectivity. Neurons and glia coexist under normal conditions to promote growth and development, A) neurons are healthy with normal morphology and both astrocytes and microglia are in resting states. During pathological (B) conditions such as chronic oxidative stress glia are activated. Activated astrocytes increase ROS production, Ca^{2+} influx, and decrease NT uptake. Activated microglia activate neighbouring microglia and sustain activation through a positive feedback loop caused by the release of microglia activators, such as laminin. These activated

microglia up-regulate and release pro-inflammatory cytokines such as TNFα, as well as increase ROS production and iNOS resulting in the overproduction of NO causing a cytotoxic state. These effects mediate the transition of the neuron into a neurotoxic state by causing loss of membrane potential, increased Ca^{2+} influx, spontaneous NT release leading to excitotoxicity, and mitochondrial dysfunction (denoted as a shift is mitochondrial colour from orange to red). A neurotoxic state results in neuronal degeneration, including reduced axon length, dendritic length, and spine density, leading to decreased neurotransmission and poor connectivity. Ca^{2+}- calcium, iNOS- inducible nitric oxide synthase, NO- nitric oxide, NT- neurotransmitter, ROS- reactive oxygen species. (Modified from Cobb & Cole, 2015.) [94]

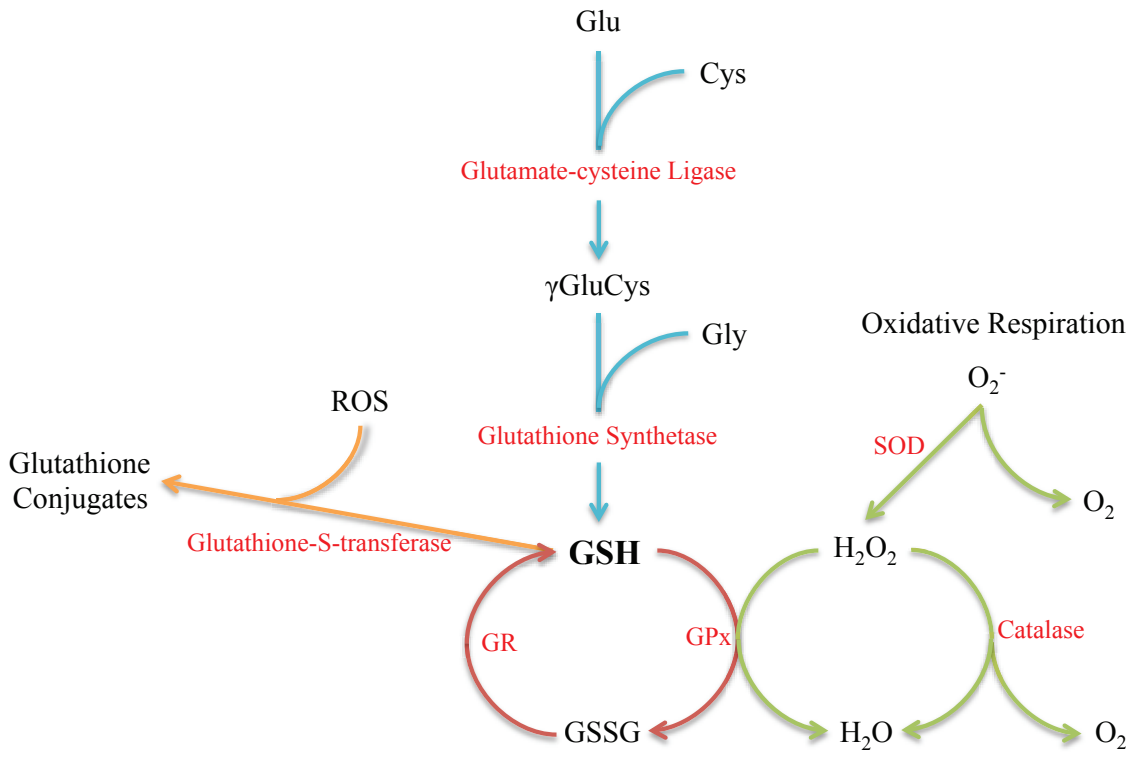

Figure 2: Metabolic Pathways of Glutathione Synthesis, Recycling, and ROS Detoxification

Glutathione synthesis is shown by the blue arrows, red arrows depict recycling, orange show detoxification, and green represent ROS production and detoxification pathways using endogenous antioxidant enzymes. Glu- Glutamate, Cys- Cysteine, γGluCys- γ-glutamylcysteine, Gly- Glycine, GSH- Glutathione, GSSG- Oxidized glutathione, ROS- Reactive oxygen species, O_2^-- Superoxide, O_2- oxygen, H_2O- Water, H_2O_2- Hydrogen peroxide, GR- Glutathione reductase, GPx- Glutathione peroxidase, SOD- Superoxide dismutase.

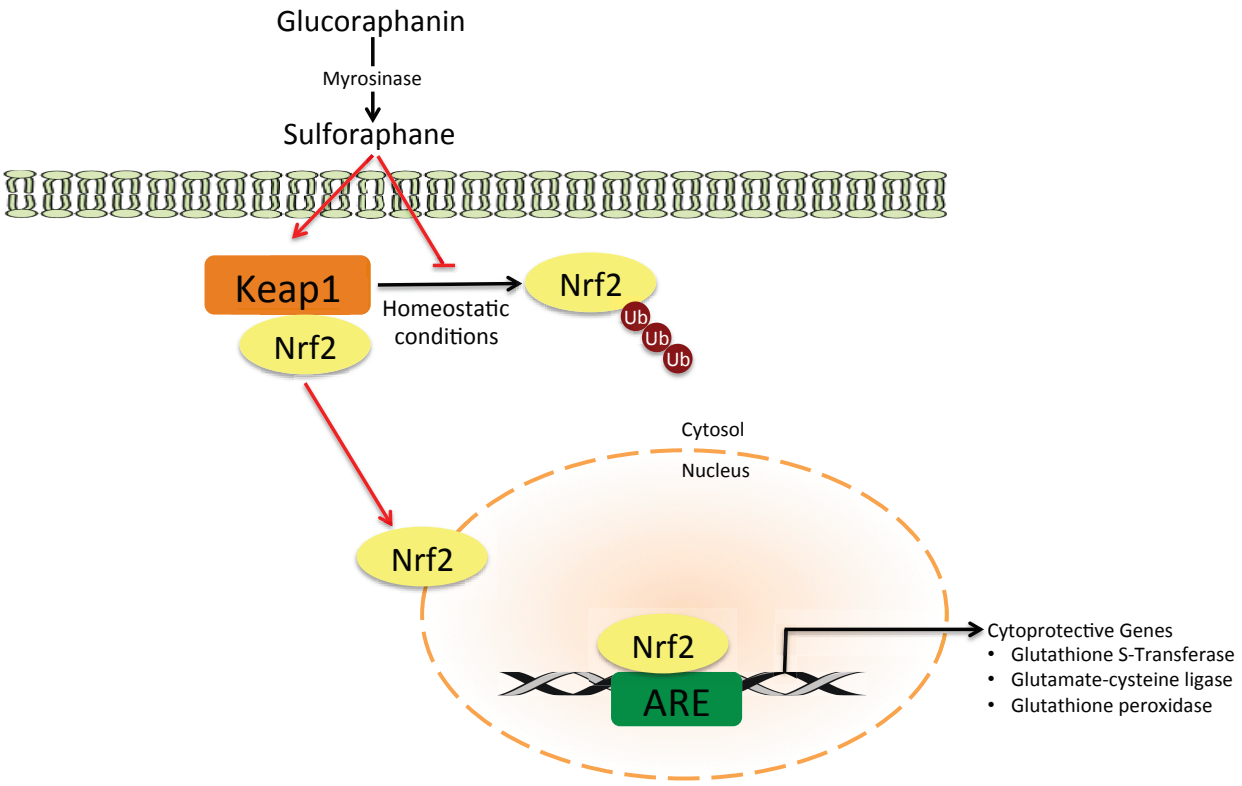

Figure 3: Mechanism of Nrf2 Mediated Cytoprotective Gene Production Induced by Sulforaphane

Under homeostatic conditions the sensor protein Keap1 sequesters the transcription factor Nrf2 by presenting it for proteosomal degradation via ubiquitination. Mechanical breakdown of broccoli sprouts combines the glucosinolate glucoraphanin with the enzyme myrosinase producing the isothiocyanate sulforaphane. In the presence of sulforaphane, Keap1 Cys residues are modified altering its ability to repress Nrf2. Nrf2 is thereby released from Keap1 and is free to translocate into the nucleus and bind to the AREs on enzyme promoter regions of DNA. This initiates the transcription of cytoprotective proteins typically known as antioxidant or detoxification enzymes. ARE – Antioxidant response element, Keap1- Kelch-like ECH-associated Protein 1, Nrf2 - Nuclear Factor E2-related Factor 2, Ub - ubiquitin.

4 MATERIALS AND METHODS

4.1 Animals and Housing Procedures

All procedures were performed in accordance with the Canadian Council on Animal Care guidelines. The Health Sciences Animal Care and Use Committee at the University of Alberta granted ethics approval for all procedures. Nulliparous female Long-Evans rats, approximately 14 weeks old (Charles River Laboratories), were timed-pregnant following an acclimation and handling period of 5 days. A methodological timeline for the experiment is shown in Figure 4. The first day of gestation (GD1) was determined by a vaginal smear containing sperm. After positive vaginal lavage, pregnant dams were randomly assigned to surgical conditions, bilateral uterine artery ligation (BUAL) or Sham surgery, as well as treatment, BrSp supplemented or standard chow diet.

Rat dams were held in a standard housing enclosure until spontaneous delivery on GD23. Rat pups were weighed and sexed on the day of birth, post-natal day 1 (P1), and culled to a litter size of 10 where more than 10 pups were born. A minimum litter size was set at 4 pups to eliminate the implications of variable litter size on outcomes. On P21 pups were weaned and held in standard Airat enclosures with two pups per cage. All animals were housed in the Health Sciences Laboratory Animal Facility at the University of Alberta, and maintained on a 12-hour light/dark cycle with food and water access ad libitum throughout the study.

4.2 Surgical Procedures

Pregnant dams underwent Sham or BUAL surgery on day 20 of a 23-day gestation. Based on the comparison of rat and human brain development by Semple et al. the timing of the injury in this model is equivalent to a preterm human infant. [71] Dams weighed between 300-400g on the day of surgery, and did not experience any unexpected complications (n=29). PI was induced using a uterine artery ligation model adapted from Wigglesworth, which was previously described by Black et al. and is depicted in Figure 5. [74,76] Briefly, dams were anesthetized using isofluorane (4% induction, 2.5% maintenance) in balanced oxygen-nitrogen and a 4cm vertical incision was made at lower midline on the shaved and sterilized abdomen. The uterine artery bifurcation was then exposed with the partial removal of both uterine horns. Once exposed, the uterine arteries were permanently ligated using 4-0 Vicryl coated sutures (Ethicon Inc., Somerville, NJ, USA) on either side of the uterine bifurcation. Tissue moisture was maintained with frequent application of sterile saline. The uterine horns were then carefully replaced inside the abdomen, followed by the suturing of the muscle layer with 4-0 Vicryl, the application of 0.2 ml bupivacaine (5mg/ml Marcaine by Hospira Healthcare Corp., Saint-Laurent, QC, Can.) for analgesia purposes, and closure of the skin with 5-0 silk (Ethicon Inc., Somerville, NJ, USA). Dams were placed back in their home cage and monitored for 8 hours to ensure full recovery. Sham operated dams underwent identical procedures, with the exception of uterine horn externalization and artery ligation. Dams delivered vaginally on GD23, and reared pups until weaning on P21. All surgical procedures were performed according to aseptic technique.

4.3 Intrauterine Growth Restriction

IUGR is clinically defined as a birth weight less than the 10th percentile or 2 standard deviations below the average weight for gestational age. Therefore for this study, IUGR was defined as less than 2 standard deviations below the mean weight of naïve litters. [236] Previously, 4 naïve litters were collected to determine the mean birth weights for our breeding colony, and thereby define the criteria of IUGR (mean= 6.28g, SD=0.38g, IUGR= ≤5.52g). [76] For the purposes of this study offspring born to BUAL operated dams weighing ≤5.52g at birth, and offspring weighing between 5.53- 7.04g from Sham operated dams were included. Weights of the offspring were taken on the day of birth, P7, and P35.

4.4 Broccoli Sprouts Preparations and Supplementation

Dams randomly allocated to receive BrSp dietary supplementation were given 200mg a day from GD15 until P21, in addition to their regular chow. Previously, it was determined that 200mg of BrSp supplemented to the regular diet provided the equivalent of a 500µg dose of SFN to the fetus, and has been shown to have protective effects in offspring. [76,227] Calabrese cultivar broccoli seeds (Mumm's seeds and sprouting, Shellbrook, SK, Canada) were selected due to the high concentration of the glucosinolate, glucoraphanin. [227] These seeds were sprouted according to supplier instructions, and air-dried for 7 days at room temperature. If the BrSp were not consumed for two consecutive days, the dam was excluded from the study.

4.5 Golgi-Cox Analysis

4.5.1 Tissue Collection and Staining Procedures

On P35, animals were anesthetized using 5% isoflourane in medical air and decapitated. After sacrifice, brain tissue was collected, cut coronally near the Circle of Willis using a brain matrix, and immersed in ~20ml Golgi-Cox fixative solution. The Golgi-Cox fixative consisted of three solutions, 5% potassium dichromate ($K_2Cr_2O_7$, Sigma 207802), 5% mercuric chloride ($HgCl_2$, Sigma 215465), and 5% potassium chromate (K_2CrO_4, Sigma 216615). All solutions were prepared in distilled water, unless otherwise specified.

After 14 days in the fixative, the tissue was transferred into a 20% sucrose solution (Sucrose (S5-3 Fisher Scientific), in 0.05M PBS) for a further 14 days at 4°C. The tissue was then cut into 200µm sections using a Leica 1000S Vibratome™, pressed onto 2% gelatinized slides, and placed in a dark humidity chamber. The 2% gelatinized slides were made by combining a 2% gelatin (G8-500 Fisher Scientific) solution with 6% chromium potassium sulfate solution (194031, ICN Biomedicals Inc.), then dipping Fisherbrand® Superfrost Plus microscope slides (12-550-15, Fisher Scientific) into the mixture, and allowing to dry overnight at 60°C.

After ≥12 hours in the humidity chamber, the tissue was processed using a Golgi-Cox staining procedure modified from Kolb & Gibb (1998).[237] All stages of this process were kept in low light or darkness until dehydration phase of staining. The tissue was dehydrated in 50% ethanol before being immersed in 28% ammonium hydroxide (NH_4OH, Sigma 320145) for ten minutes in the dark. After a brief wash in distilled water the slides were submerged in a 5% sodium thiosulfate solution ($Na_2S_2O_3$, Sigma 217263) for a further ten minutes. The slides were

then washed with distilled water, and dehydrated before being placed into xylene. The slides were cover-slipped using permount (Fisher SP15-100) and left to dry in low light for a week. Slides were coded to ensure blinding during analysis.

4.5.2 Cortical Thickness Measurements

Whole brain Golgi-Cox stained sections were photographed using a Leica GZ6E stereoscope set at a magnification of 0.67 X. Cortical thickness measurements were taken at three points at each of three planes; from the edge of the cortex to the edge of the white matter using ImageJ 64 software, adapted from Stewart and Kolb.[238] These three cortical measures were made at points medial, central, and lateral on three sections of the tissue, as illustrated in Figure 6. Measurements were taken from one hemisphere, which was selected at random. The planes of these sections were identified by the following landmarks described by Zilles: Plane 1: First section where the rhinal fissure does not transect the brain and the forceps minor is visible. Plane 2: Anterior commissure visible. Plane 3: First hippocampal section where fully formed and dentate gyrus separated from the subiculum.[239] The mean cortical thickness in millimeters was calculated by averaging across the measures and planes.

4.5.3 Dendritic Analyses

Slides were coded to blind the experimenter to animal group. The regions of interest for this study included the CA1 region of the hippocampus (Figure 7B), and layer V of the hind limb primary motor cortex (M1) (Figure 7A), as defined by Zilles (1985).[239] These regions were chosen for analysis as IUGR offspring from this model have shown robust motor impairments in

their hind limbs as well as significant abnormalities in learning and memory tasks [manuscript in progress]. [20] The CA1 region is known for its role in memory and is particularly vulnerable to hypoxic-ischemic insults. [240] Layer V of the primary motor cortex is responsible for integrating and relaying information from the cortex to the brainstem and spinal cord functionally executing movements, in conjunction with other motor areas such as the premotor cortex. [241,242] The tracts that are formed from the axons of these cells and responsible for the execution of movement are the corticobulbar and corticospinal. [242] To meet criteria for analysis, the entirety of the dendritic tree had to be intact and well impregnated. Cells were not used if obscured by blood vessels, astrocytes, adjacent cells, or stain precipitations. Two forms of analysis were used to quantify the dendritic morphology. First, Sholl analysis (a transparent grid of concentric, equidistant circles equivalent to 11.11μm apart placed over the cell body) was performed as an estimation of DL, where the number of dendritic intersections on each overlain concentric ring was counted to calculate mean length. [243] Second, dendritic complexity was estimated using the number of branch bifurcations and classifying them into branch order according to Coleman & Riesen. [244]

Neuronal tracings of Golgi-Cox stained pyramidal cells were made using a camera lucida (total magnification: 200X). Ten neurons (5/hemisphere) in each region of interest were traced from each animal (Sham (n=12), Sham+BrSp (n=12), IUGR (n=12), IUGR+BrSp (n=12)). The mean of the ten cells per brain region for dendritic measures were used as the unit of analysis.

4.5.4 Spine Analyses

Third order or higher terminal branches of dendrites were drawn at 1000x magnification using a camera lucida. Both apical and basilar dendrites in the CA1 region of the hippocampus

(Figure 7B), and basilar dendrites from layer V of the M1 region (Figure 7A) (10 neurons (5/hemisphere)/region) were drawn. After identification of dendrites the total number of visible spines were counted. Spine density was expressed per 10μm after the total length of the drawn segment was calculated using ImageJ64 Software. Segments were selected if they were well impregnated and were not obscured by blood vessels or adjacent cells.

4.6 Statistical Analysis

All analyses were performed using SPSS statistical software (SPSS Statistics 17.0; SPSS Inc., Chicago, IL, USA), with the exception of the power calculations (80%), which was conducted using Stata 10 software (StataCorp. 2007. Stata Statistical Software: Release 10. Colledge Station, TX: StataCorp LP.). A sample size of 6 animals/sex/group was calculated for the DL and branch points (BPs) analyses, resulting in a total required sample size of 48 animals. For spine density measures, a sample size of 5 animals/sex/group was calculated resulting in a total required sample size of 40 animals. All outcome variables were compared by three-way factorial analysis of variance (ANOVA), with the exception of offspring viability, which was analyzed using a two-way factorial ANOVA. The independent factors for analysis compared by three-way ANOVA, were 'surgery' (IUGR) (2), 'sex' (2), and 'treatment' (BrSp) (2). In the two-way ANOVA analysis of offspring viability, maternal conditions of 'surgery' (IUGR) (2) and 'treatment' (BrSp) (2) were used as independent factors.

All sample data was first assessed for normality and equality of variances between the groups of the levels of factors using Shapiro-Wilks and Levene's tests, respectively. Adjusted p-values were reported if the sample data violated the Levene's test. Pair-wise independent t-test(s)

were conducted post-hoc when significant two-way interactions were detected. Family-wise alpha was defined as $p<0.05$. The data is expressed as mean ± standard error of the mean (SEM) in the graphs and tables below, and significance was denoted as follows: * main effect of surgery, # main effect of sex, + main effect of diet, ‡ interaction between surgery and diet, § interaction between surgery and sex.

5 METHODS FIGURES

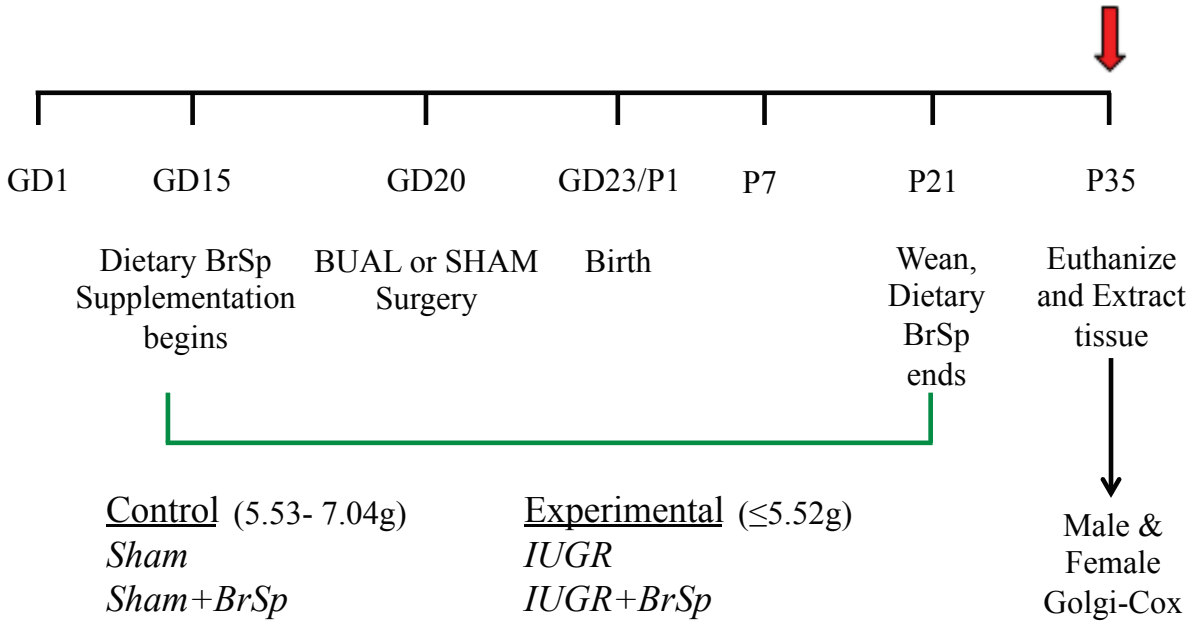

Figure 4: Methodological Timeline for Experiment

The first day of pregnancy was denoted as gestational day (GD) 1. Dams randomly allocated to receive BrSp supplementation began on GD15 until postnatal day (P)21 when the pups were weaned. On GD20 dams were randomly selected to undergo either Sham or BUAL surgery. Offspring were naturally born on GD23, also known as P1. On P7 offspring were weighed and ears notched for later identification. Offspring were euthanized on P35 to extract brain tissue and carry out Golgi-Cox experimentation.

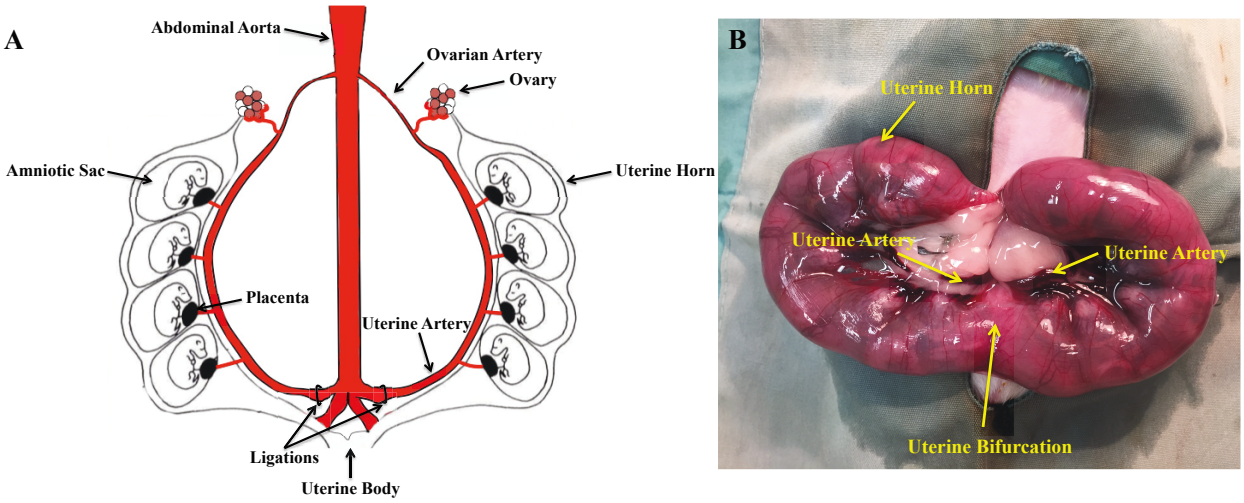

Figure 5: Representations of Bilateral Uterine Artery Ligation

This figure depicts the surgical procedure rat dams underwent as a model of placental insufficiency. A) Schematic of the location of bilateral ligations of the uterine artery on either side of the uterine bifurcation. B) Image of the anatomy of the rat uterus through externalization during surgery.

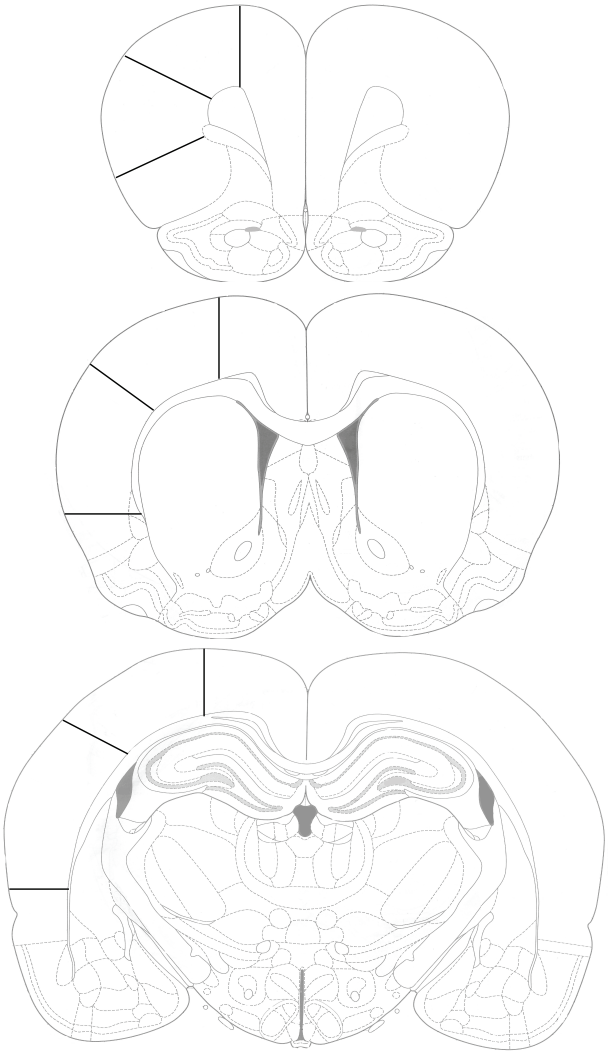

Figure 6: Schematic Sections of Cortical Thickness Measurements

Three measurements, lateral, medial, and central, were taken at each plane, indicated by the thick black lines. Measurements were taken from one hemisphere, which was selected at random. The planes were identified according to Zilles (1985) nomenclature: Plane 1: First section where the rhinal fissure does not transect the brain and the forceps minor is visible. Plane 2: Anterior commissure visible. Plane 3: First hippocampal section where fully formed and dentate gyrus separated from the subiculum. [239]

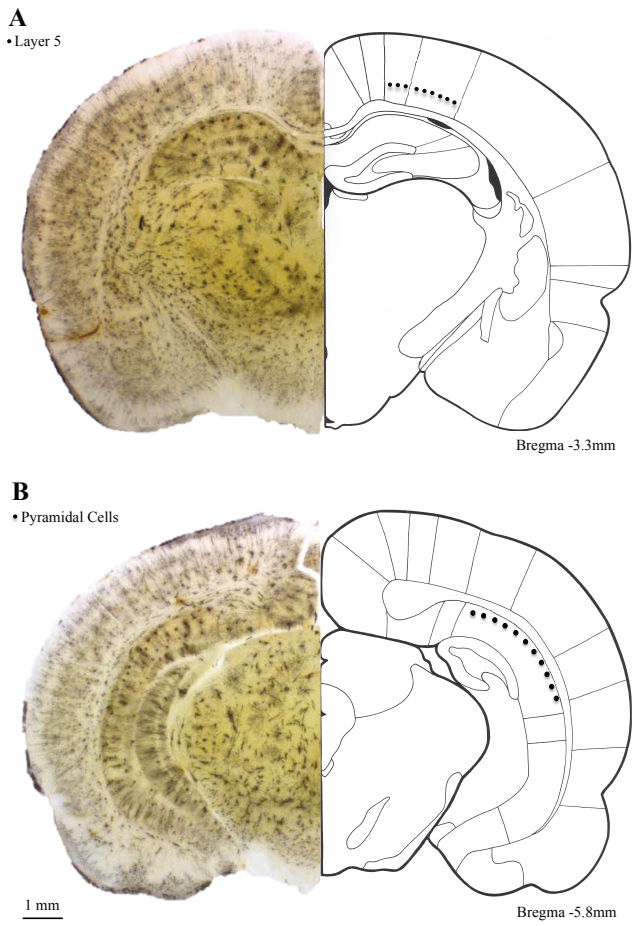

Figure 7: Schematic Illustration of the Areas of Golgi-Cox Analysis

Five pyramidal neurons per hemisphere were analyzed in each region for all animals. The cortical areas were defined according to Zilles (1985). [239] (A) Neurons and spines were traced from layer V of the hindlimb primary motor cortex, indicated by the black dots. (B) Neurons and spines were traced from a posterior section of the CA1 region of the hippocampus, indicated by the black dots.

6 RESULTS

Data from 71 offspring was collected (Sham=20, Sham+BrSp=16, IUGR=18, IUGR+BrSp=17). A minimum of five dams were utilized per group to control for maternal effects on outcomes (Sham=6, Sham+BrSp=5, IUGR=7, IUGR+BrSp=11). Due to staining variability and neuron inclusion criteria, we were unable to utilize all of the same offspring for each region of analysis; therefore additional animals were collected to reach statistical power.

6.1 Offspring Viability and Weights

Offspring viability assessed the total number of live offspring born to each dam in this study, to determine whether BrSp supplementation affected fetal mortality (Figure 8A). A significant main effect of surgery was found ($F(1, 24)= 36.62$, $p<0.001$), showing that significantly less viable offspring were born to IUGR dams, regardless of BrSp supplementation. This indicates that BrSp did not affect fetal mortality in this model.

Body weights of offspring were recorded at several time points to determine changes in growth. Analysis of offspring weights, who met inclusion criteria, at birth ($F(1,57)= 131.97$, $p<0.001$), P7 ($F(1,57)= 46.63$, $p<0.001$), and P35 ($F(1,57)= 27.15$, $p<0.001$) , showed a significant main effect of surgery (Figure 8B, 8C, 8D, respectively). IUGR offspring had significantly lower body weights at all time points, with no impact of BrSp supplementation, indicating that offspring did not catch up in growth. A significant main effect of sex was also found at P7 ($F(1,57)=4.29$, $p=0.043$), and P35 ($F(1,57)=42.79$, $p<0.001$) depicting the known growth differences between male and female body weights. As shown in supplementary figure 1

of the Appendix, birth weights of all animals, including those that did not meet study criteria, were only found to have a main effect of surgery ($F(1,162)=256.51$, $p<0.001$) and a main effect of sex ($F(1,162)=15.70$, $p<0.001$). Therefore, our selection criteria did not exclude a positive effect of BrSp on birth weight in this study.

6.2 Cortical Thickness

Cortical thickness was analyzed as a rough morphometric measure of neuron number. [245] Interestingly, no differences in mean cortical thickness were found between the four groups (Figure 9). This roughly indicates that the number of cells in the cortex is similar across groups in this study. Changes in neuronal morphology may be matched by a compensatory increase in glial cell numbers, leading to the lack of differences in cortical thickness.

6.3 Neuronal Morphology

Neuronal morphology was analyzed to explore the underlying pathology of IUGR in relation to NDD and determine if BrSp supplementation impacts the structure and ultimately the function of these vital cells.

6.3.1 CA1 region of the Hippocampus

Representative neurons from each of the four groups in the CA1 region are shown in Figure 10. As shown in Figure 11A, BrSp significantly increased basilar DL in IUGR offspring in the CA1 region. A significant two-way interaction was found between surgery and diet ($F(1,$

40)= 18.88, p<0.001), as well as between surgery and sex (F(1,40)= 9.10, p=0.004) for basilar DL. Significant main effects were also found for diet (F(1,40)=6.15, p=0.017), surgery (F(1,40)=4.32, p=0.044), and sex (F(1,40)= 4.97, p=0.031). Post hoc analysis (Table 1) showed that supplementation with BrSp prevented basilar dendrite retraction as IUGR offspring had significantly lower basilar DL (1617.25 ± 50.69μm) compared to both Sham controls (1900.61 ± 37.88μm, t-test: p<0.001) and IUGR+BrSp offspring (1918.32 ± 61.32μm, t-test: p=0.001). Moreover, IUGR+BrSp offspring were not different from Sham+BrSp controls (1818.34 ± 45.38μm, t-test: p=0.204). Sex differences were also detected (Table 2). Male IUGR offspring (1652.06 ± 55.04μm) had significantly shorter dendrites compared to IUGR females (1883.51 ± 70.61μm, t-test: p=0.017). Additionally, male IUGR offspring had significantly less total DL than Sham males (1876.85 ± 42.01μm, t-test: p=0.004), while female IUGR were not significantly different from Sham females.

As well, a significant two-way interaction between surgery and diet (F(1,40)= 10.60, p=0.002) was found in basilar total BPs, a measure of dendritic arborization (Figure 11C). IUGR offspring on a regular chow diet (46.20 ± 0.73) had significantly less basilar BPs than IUGR offspring supplemented with BrSp (50.33 ± 1.26, t-test: p=0.010), indicating that protection against injury has been attained (Table 3). Significant differences were also found between Sham (51.13 ± 1.58) and IUGR offspring (Adjusted t-test: p=0.012, Table 3). Moreover, no significant differences were found between IUGR+BrSp offspring and Sham+BrSp controls (47.51 ± 1.19, t-test: p=0.119), further demonstrating the prevention of injury with BrSp supplementation (Table 3).

A three-way ANOVA of apical DL showed a significant two-way interaction between surgery and sex ($F(1, 40)= 8.41$, $p=0.006$) as well as main effects of surgery ($F(1,40)=7.86$, $p=0.008$) and sex ($F(1,40)=23.09$, $p<0.001$) (Figure 11B). Significant differences were found between male IUGR offspring (3029.22 ± 101.35μm) and Sham males (3603.25 ± 99.09μm, t-test: $p=0.001$) following pairwise t-test analysis (Table 4). Moreover, male IUGR offspring had significantly shorter dendrites compared to IUGR females (3804.75 ± 94.16μm, t-test: $p<0.001$, Table 4).

Similarly, a significant two-way interaction between surgery and sex ($F(1,40)=6.33$, $p=0.016$) was found for apical BPs (Figure 11D). Following the pairwise t-test male IUGR (89.05 ± 2.59) animals were found to have significantly less apical BPs than male Sham offspring (101.05 ± 3.86, t-test: $p=0.017$, Table 5). Moreover, male IUGR offspring had fewer apical BPs compared to female IUGR animals (102.08 ± 2.38, Adjusted t-test: $p= 0.001$, Table 5). Supplementation with BrSp did not impact the apical dendrites of the CA1 in controls or IUGR offspring, however a sex effect was observed. These results suggest that not only are the apical and basilar dendrites differentially impacted, but also male and female offspring receive variable levels of injury in this model in this region.

6.3.2 Primary Motor Cortex

Layer V M1 representative neurons can be found in Figure 12. As shown in Figure 13 and Figure 14B respectively, BrSp supplementation had a significant main effect on basilar DL ($F(1,40)=8.73$, $p=0.005$) and BPs ($F(1,40)=9.18$, $p=0.004$) in the M1. Although morphological changes were absent in IUGR offspring, these results indicate a potential neurological benefit for

offspring with healthy maternal dietary changes. A significant main effect of sex was also found in basilar BPs ($F(1,40)=5.22$, $p=0.028$) (Figure 14B).

6.4 Spine Density

Spine density was analyzed in both regions of analysis to understand the complete morphological changes resulting from surgery and treatment. The density of spines on dendritic segments determines the number of synapses formed and thus the amount of innervation a dendrite receives.

6.4.1 CA1 region of the Hippocampus

As shown in Figure 15, significant main effects of diet ($F(1,32)=4.28$, $p=0.047$) and surgery ($F(1,32)=11.60$, $p=0.002$) were found in the basilar dendritic spine density in the CA1 region of the hippocampus. BrSp supplementation seems to decrease synaptic density on the basilar dendrites. Interestingly, no effect of sex was found. No significant differences were found for spine density on the apical dendrites of the CA1 region (Figure 16).

6.4.2 Primary Motor Cortex

In layer V of the M1 region no significant differences were found following the three-way ANOVA. These results are shown in Figure 17.

6.5 Summary

PI induced IUGR caused morphological alterations to neurons of the CA1 region of the hippocampus, particularly in the basal arbors, further supporting the notion of functional separation of apical and basilar dendrites. Some of these alterations were prevented following maternal BrSp consumption during pregnancy and lactation. Additionally, sex differences were found with males generally more predominately affected than female offspring. This is in agreement with the clinical findings of significantly higher incidence of NDD in males. A reduction in dendritic spine density in the basilar arbors of the CA1 region was also observed in both IUGR and BrSp groups. These results may be indicating a compensatory change in spine density due to longer dendrites. IUGR did not have an effect on layer V of the M1 region, suggesting that this layer is resilient against the injury caused by this model. Interestingly, structural alterations of the neurons were observed, without changes in the cortical thickness, suggesting glial compensation or recovery. Alternatively, the subtlety of the pathological changes seen may not be large enough to result in detectable changes in cortical thickness. BrSp afforded significant neuroprevention of morphological alteration of the CA1 basilar dendrites indicating the potential of BrSp supplementation as a novel preventive intervention during gestation.

7 RESULTS FIGURES AND TABLES

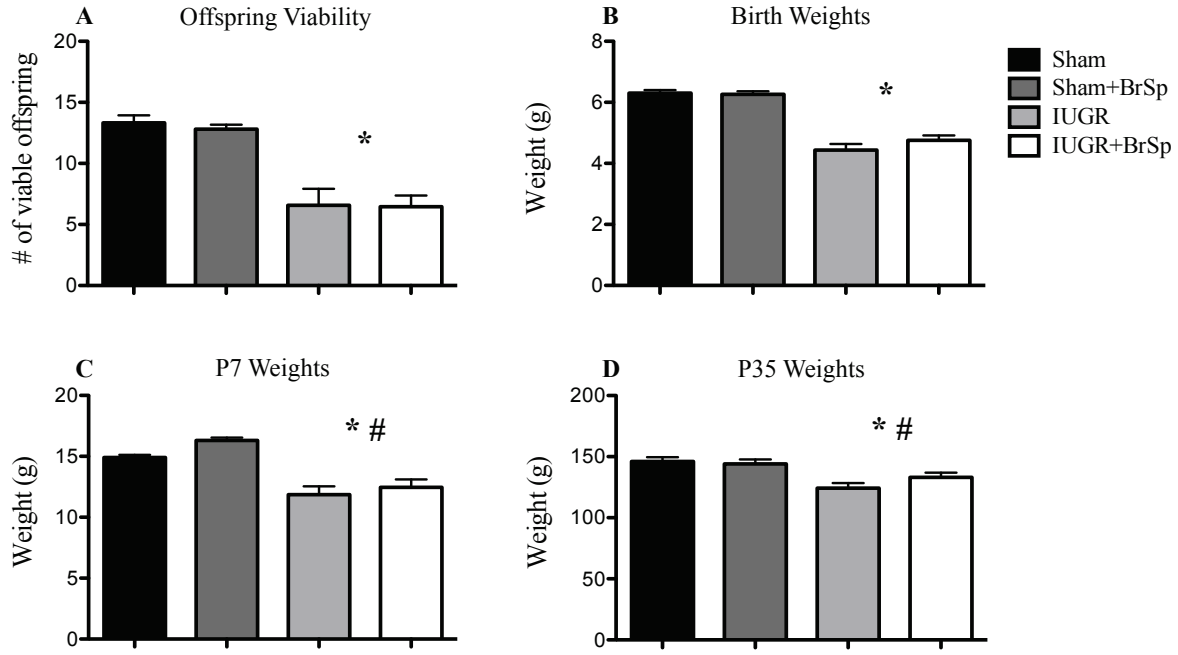

Figure 8: Offspring Viability and Weights

Offspring of both sexes were utilized in this study. The number of viable offspring (A) born to each dam (Sham n=6, Sham+BrSp n=5, IUGR n=7, IUGR+BrSp n=11) utilized in the study was counted, regardless of inclusion criteria. The weights of all offspring used in this study, which met inclusion criteria, were taken at birth (B), postnatal day 7 (C), and postnatal day 35 (D) (Sham n=18, Sham+BrSp n=14, IUGR n=16, IUGR+BrSp n=17). Due to variability in tissue staining and damage some animals could not be utilized for all areas of analysis therefore more offspring were collected to reach statistical power. Data expressed as mean ± SEM, * main effect of surgery, # main effect of sex ($p<0.05$).

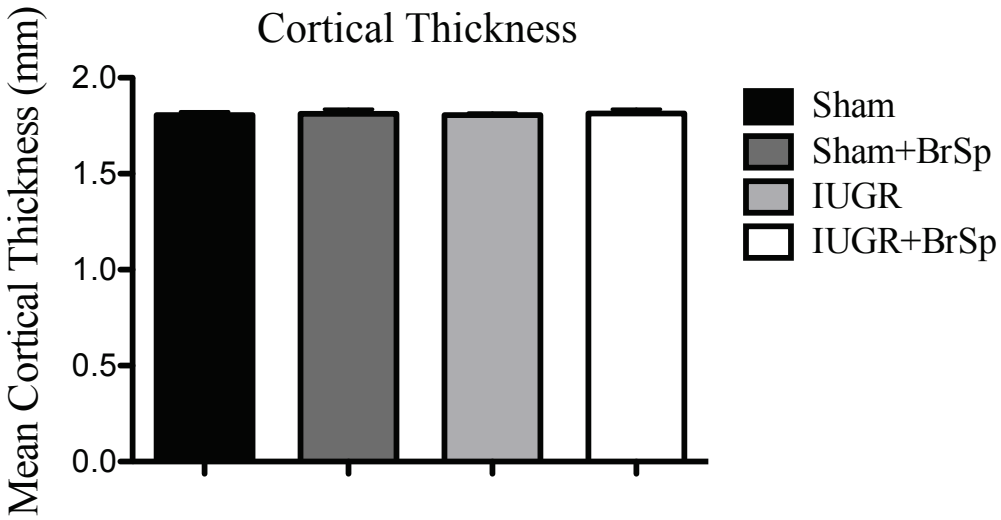

Figure 9: Cortical Thickness Measurements of the Rat Brain

Cortical thickness was measured across three planes of the rat brain through three sections. No significant differences were detected following the 3-way ANOVA (Sham n=12, Sham+BrSp n=12, IUGR n=12, IUGR+BrSp n=12). Values are shown as mean ± SEM.

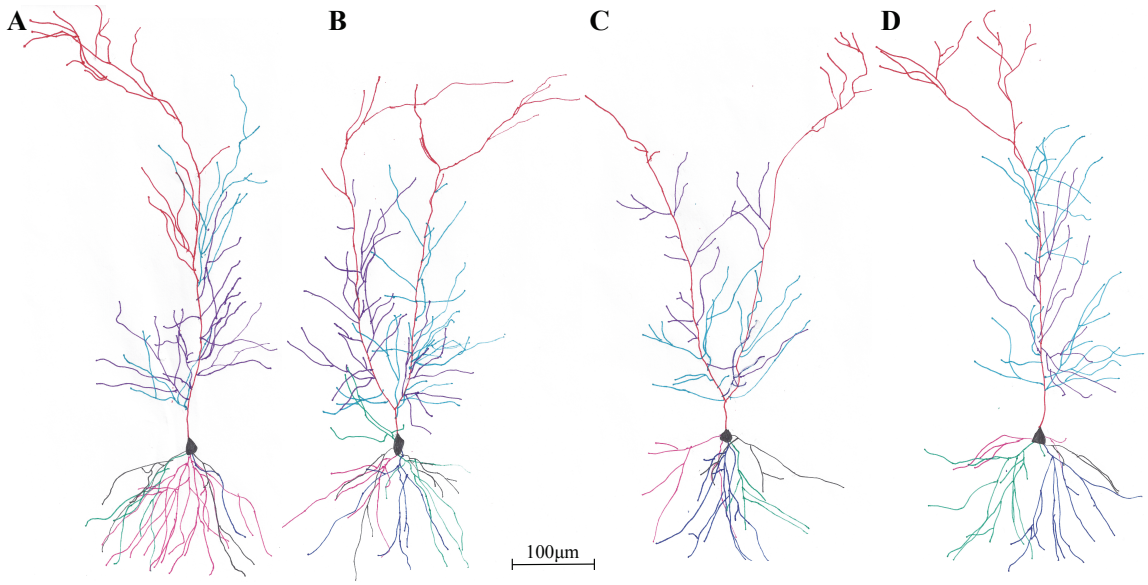

Figure 10: Drawings of Representative Neurons from the CA1 Region

Tracings of neurons from each of the four groups, Sham (A), Sham+BrSp (B), IUGR (C), and IUGR+BrSp (D), are shown. The basal end of the neuron is depicted by green, blue, pink and black dendrites, while the apical dendrites are drawn in red, purple, and aqua.

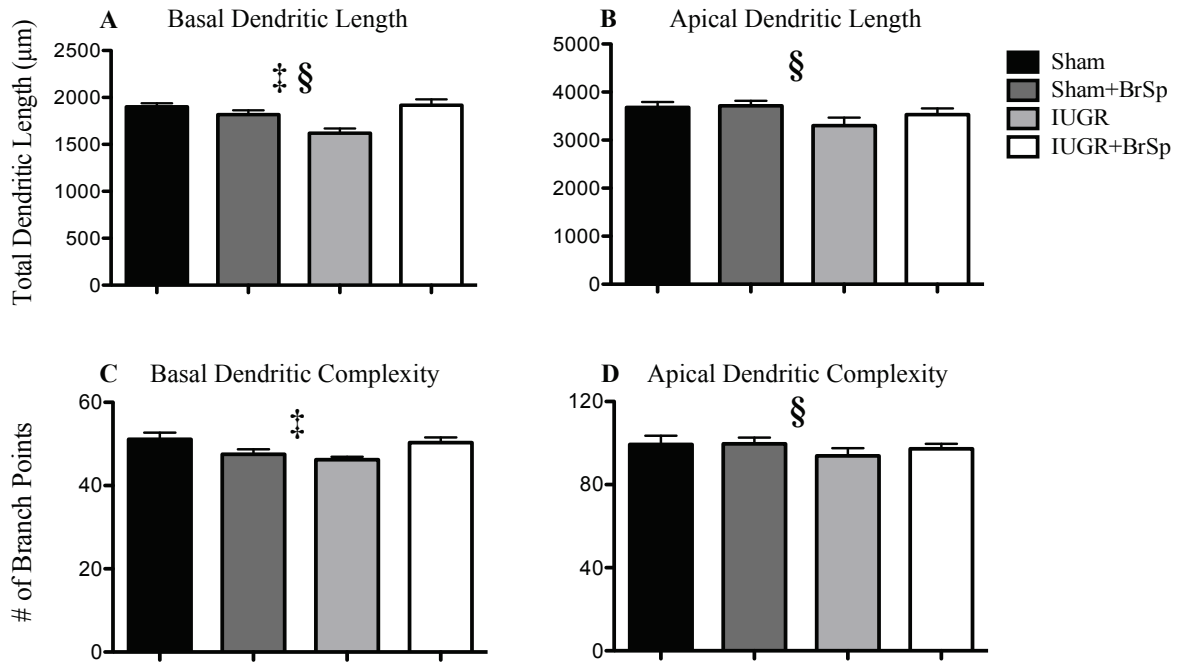

Figure 11: Quantitative Analysis of Total Dendritic Length and Dendritic Complexity in the Basal and Apical Arbors of CA1 Hippocampal Neurons

Total length of basilar (A) and apical (B) dendrites (µm) of offspring from all four groups (Sham n=12, Sham+BrSp n=12, IUGR n=12, IUGR+BrSp n=12) is shown in this figure. The total number of branch points for both basilar and apical dendrites are represented in C and D, respectively. Values are shown as mean ± SEM, ‡ interaction between surgery and diet, § interaction between surgery and sex, $p<0.05$.

Pair	Mean ± Std. Error	N	Levene's Test p-value	p- value	Adjusted p-value
Sham	1900.61 ± 37.88	12	0.712	0.178	0.178
Sham+BrSp	1818.34 ± 45.38	12			
IUGR	1617.25 ± 50.69	12	0.475	**0.001**	0.001
IUGR+BrSp	1918.32 ± 61.32	12			
Sham	1900.61 ± 37.88	12	0.269	**0.000**	0.000
IUGR	1617.25 ± 50.69	12			
Sham+BrSp	1818.34 ± 45.38	12	0.209	0.204	0.205
IUGR+BrSp	1918.32 ± 61.32	12			

Table 1: Pairwise t-test Comparison of CA1 Basilar Dendritic Length for Surgery vs Diet Interaction

This table shows results from the Pair-wise independent t-test from basilar dendritic length (μm) data, reported as mean ± SEM. Significant differences were found between IUGR and IUGR+BrSp, as well as Sham and IUGR offspring ($p<0.05$), shown in bold.

Pair	Mean ± Std. Error	N	Levene's Test p-value	p- value	Adjusted p-value
Sham- Male	1876.85 ± 42.01	12	0.766	0.576	0.576
Sham- Female	1842.09 ± 44.53	12			
IUGR- Male	1652.06 ± 55.04	12	0.259	**0.017**	0.017
IUGR- Female	1883.51 ± 70.61	12			
Sham- Female	1842.09 ± 44.53	12	0.066	0.625	0.626
IUGR- Female	1883.51 ± 70.61	12			
Sham- Male	1876.85 ± 42.01	12	0.573	**0.004**	0.004
IUGR- Male	1652.06 ± 55.04	12			

Table 2: Pairwise t-test Comparison of CA1 Basilar Dendritic Length for Surgery vs Sex Interaction

This table shows results from the Pair-wise independent t-test from basilar dendritic length (μm) data, reported as mean ± SEM. Significant differences were found between IUGR-Males and IUGR-Females, as well as Sham-Males and IUGR-Males ($p<0.05$), shown in bold.

Pair	Mean ± Std. Error	N	Levene's Test p-value	p- value	Adjusted p-value
Sham	51.13 ± 1.58	12	0.201	0.082	0.083
Sham+BrSp	47.51 ± 1.20	12			
IUGR	46.20 ± 0.73	12	0.098	**0.010**	0.011
IUGR+BrSp	50.33 ± 1.26	12			
Sham	51.13 ± 1.58	12	0.007	0.010	**0.012**
IUGR	46.20 ± 0.73	12			
Sham+BrSp	47.51 ± 1.20	12	0.839	0.119	0.119
IUGR+BrSp	50.33 ± 1.26	12			

Table 3: Pairwise t-test Comparison of CA1 Basilar Dendritic Complexity for Surgery vs Diet Interaction

This table shows results from the Pair-wise independent t-test from basilar branch points data, reported as mean ± SEM. Significant differences were found between IUGR and IUGR+BrSp, as well as Sham and IUGR offspring ($p<0.05$), shown in bold.

Pair	Mean ± Std. Error	N	Levene's Test p-value	p- value	Adjusted p-value
Sham- Male	3603.25 ± 99.09	12	0.750	0.206	0.207
Sham- Female	3794.94 ± 108.91	12			
IUGR- Male	3029.22 ± 101.35	12	0.677	**0.000**	0.000
IUGR- Female	3804.75 ± 94.16	12			
Sham- Female	3794.94 ± 108.91	12	0.316	0.946	0.946
IUGR- Female	3804.75 ± 94.16	12			
Sham- Male	3603.25 ± 99.09	12	0.744	**0.001**	0.001
IUGR- Male	3029.22 ± 101.35	12			

Table 4: Pairwise t-test Comparison of CA1 Apical Dendritic Length for Surgery vs Sex Interaction

This table shows results from the pair-wise independent t-test from apical dendritic length (μm) data, reported as mean ± SEM. Significant differences were found between IUGR- Males and IUGR- Females, as well as Sham- Male and IUGR- Male offspring ($p<0.05$), shown in bold.

Pair	Mean ± Std. Error	N	Levene's Test p-value	p- value	Adjusted p-value
Sham- Male	101.05 ± 3.86	12	0.424	0.550	0.550
Sham- Female	97.97 ± 3.30	12			
IUGR- Male	89.05 ± 2.59	12	0.895	**0.001**	0.001
IUGR- Female	102.08 ± 2.38	12			
Sham- Female	97.97 ± 3.30	12	0.303	0.323	0.324
IUGR- Female	102.08 ± 2.38	12			
Sham- Male	101.05 ± 3.86	12	0.093	**0.017**	0.018
IUGR- Male	89.05 ± 2.59	12			

Table 5: Pairwise t-test Comparison of CA1 Apical Dendritic Complexity for Surgery vs Sex Interaction

This table shows results from the Pair-wise independent t-test from apical branch points data, reported as mean ± SEM. Significant differences were found between IUGR- Males and IUGR-Females, as well as Sham- Males and IUGR- Males ($p<0.05$), shown in bold.

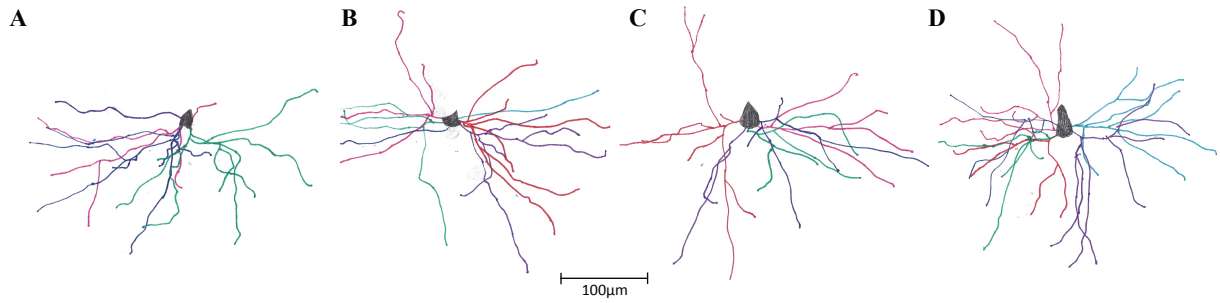

Figure 12: Drawings of Representative Neurons from Layer V of the Primary Motor Cortex

Tracings of the basilar tree from each of the four groups are shown, Sham (A), Sham+BrSp (B), IUGR (C), and IUGR+BrSp (D).

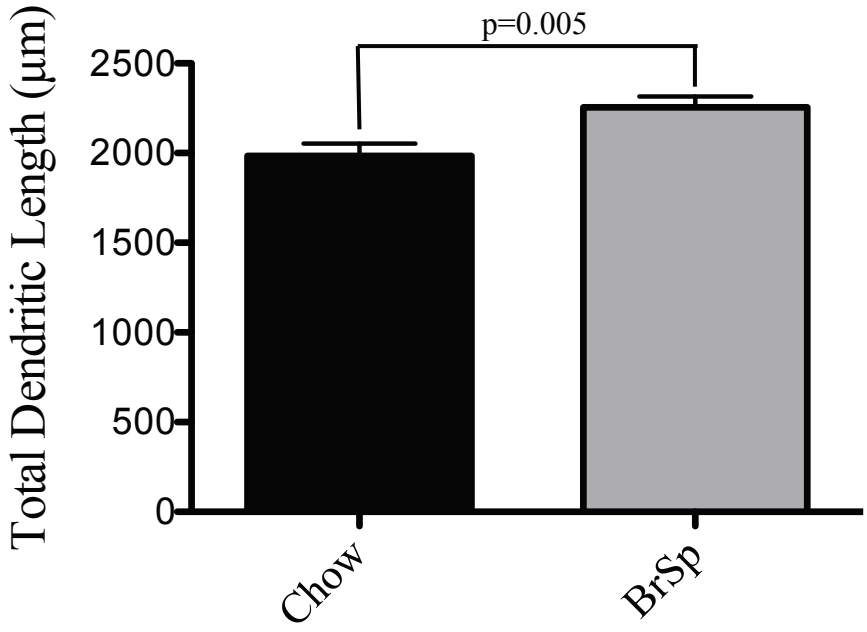

Figure 13: Quantitative Analysis of Overall Dendritic Length in Basal Dendrites of Layer V Primary Motor Cortex Neurons

The average length of basilar dendrites, measured in µm, is shown for all offspring (Sham n=12, Sham+BrSp n=12, IUGR n=12, IUGR+BrSp n=12). A significant main effect of diet was found (Chow n=24, BrSp n=24). Values are shown as mean ± SEM.

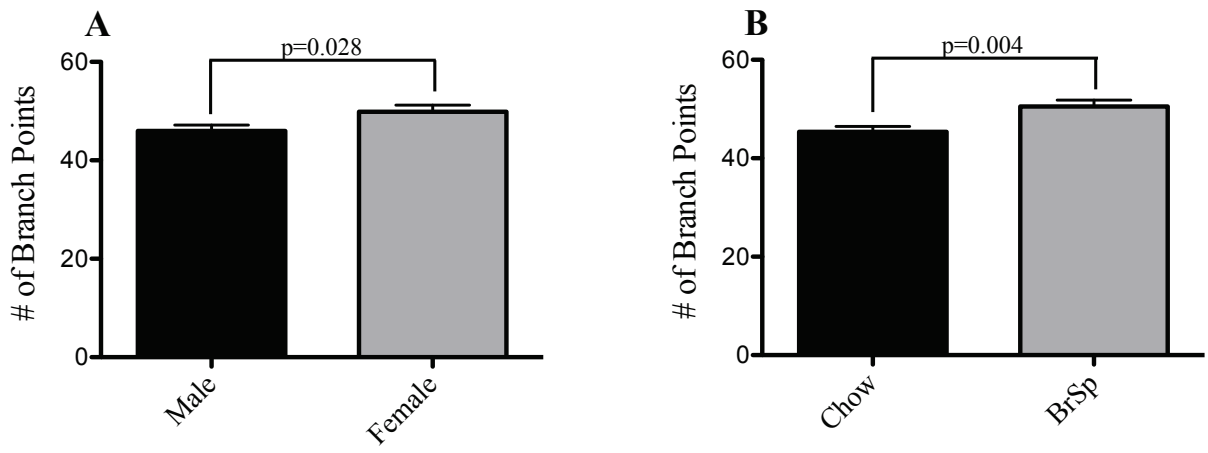

Figure 14: Quantitative Analysis of Overall Dendritic Complexity in Basal Dendrites of Layer V Primary Motor Cortex Neurons

Basilar dendritic complexity calculated by total number of branch points along the dendrites is shown for all offspring (Sham n=12, Sham+BrSp n=12, IUGR n=12, IUGR+BrSp n=12). Significant main effects of sex (A) (Male n=24, Female n=24) and diet (B) (Chow n=24, BrSp n=24) were found. Values are shown as mean ± SEM.

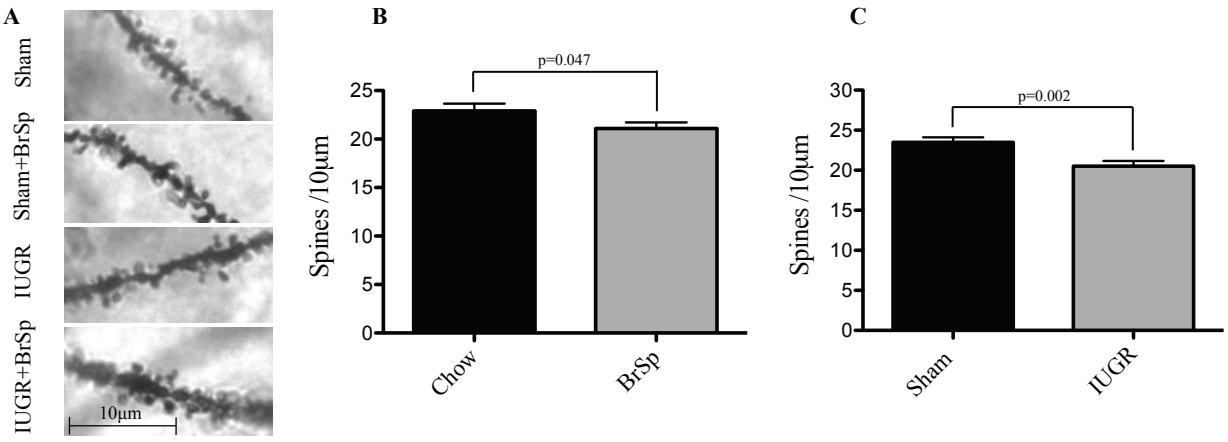

Figure 15: Spine Density Analysis for Basilar CA1 Dendrites

The average spine density of basilar dendrites (/10µm), is shown for all offspring (Sham n=12, Sham+BrSp n=12, IUGR n=12, IUGR+BrSp n=12) in B. Representative images of basilar dendritic segments taken at 1000X magnification used for spine density analysis are shown in A. Significant main effects of diet (B) (Chow n=24, BrSp n=24) and surgery (C) (Sham n=24, IUGR n=24) were found on the basilar dendrites. Values are shown as mean ± SEM.

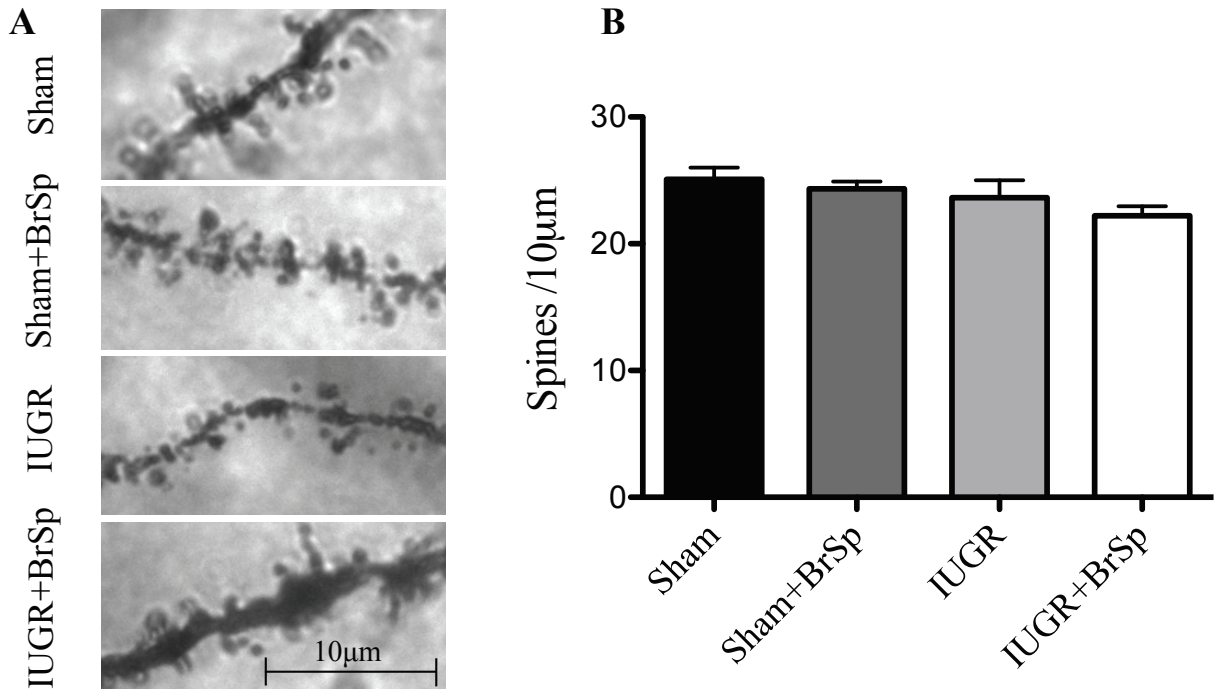

Figure 16: Spine Density Analysis for Apical CA1 Dendrites

Images of representative dendritic segments taken at 1000X magnification, shown in A were used for spine density analysis. The average spine density of apical dendrites (/10µm) is shown for all offspring (Sham n=12, Sham+BrSp n=12, IUGR n=12, IUGR+BrSp n=12) in B. No significant differences were found between the groups. Values are shown as mean ± SEM.

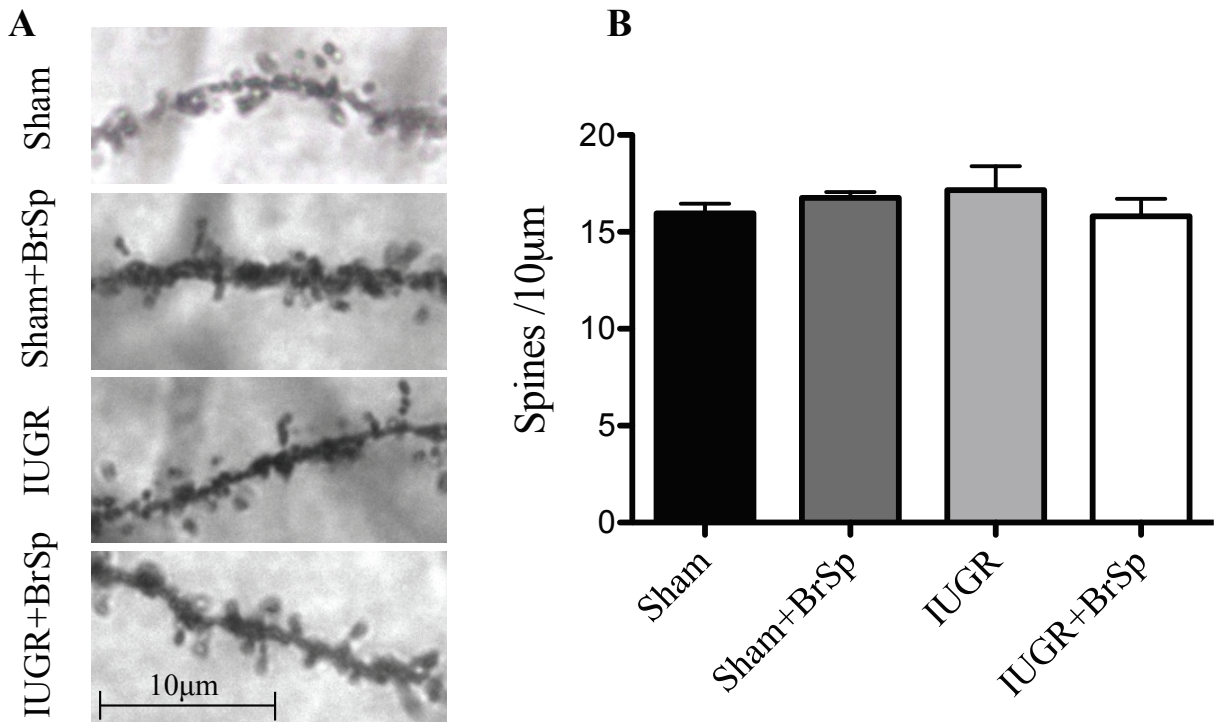

Figure 17: Spine Density Analysis for the Primary Motor Cortex

The average spine density of basilar (B) dendrites are shown /10µm for all offspring (Sham n=12, Sham+BrSp n=12, IUGR n=12, IUGR+BrSp n=12). Images of representative dendritic segments used for spine density analysis taken at 1000X magnification are shown in A. No significant differences were found between the four groups following a 3-way ANOVA. Values are shown as mean ± SEM.

8 DISCUSSION

Abnormal brain development caused by a suboptimal intrauterine environment can result in NDD manifestation. PI and IUGR have been associated with fetal oxidative stress, which adversely affects brain development. [79,246] Unfortunately, IUGR affects up to 30 million newborns a year making it one of the leading causes of perinatal morbidity and mortality. [47-50] Worse still, there are currently no interventions to prevent antepartum insults such as IUGR. The need of a preventive intervention has increased due, in part, to the fact that the majority of insults resulting in NDD occur during gestation, and improved survival rates for IUGR infants. [29] SFN, the isothiocyanate metabolite of BrSp, has shown positive benefits in several areas of research, including previous work by our laboratory on this model [manuscript in progress]. [20,76,222-224] However, to our knowledge the effect of maternal dietary BrSp supplementation on offspring neuronal morphology has not been explored in a model of PI. The function of the nervous system depends on the correct growth, arborization, and integration of billions of neurons into a neural network. Dendritic morphology is critical for network formation. [177,178] In this regard, the purpose of the present study was to examine the neuronal morphological changes associated with IUGR and the impact of BrSp as a novel neuropreventive therapy. BrSp contain many naturally bioactive substrates, including SFN precursors, which combat OS through antioxidant and anti-inflammatory mechanisms. Therefore, it was hypothesized that these properties would prevent injury to the developing brain when supplemented during pregnancy and lactation.

This study has demonstrated that PI induced IUGR during the end of gestation, which coincides with rapid hippocampal growth, neuronal differentiation, gliogenesis, and synaptogenesis, leads to marked alterations in dendritic morphology of CA1 pyramidal neurons,

particularly the basilar arbors. [71] As expected, these changes in CA1 morphology were, for the most part, prevented with BrSp supplementation. In addition, it was found that BrSp supplementation influenced the DL and complexity of layer V M1 neurons. Overall, male offspring were more significantly impacted by IUGR than females. These changes are likely responsible for at least some of the observed behavioural improvements seen in BrSp fed IUGR animals in our previous work [manuscript in progress]. [20,76]

8.1 Experimental Procedures

BUAL was utilized in this study as a model of PI, which has consistently induced asymmetrical IUGR in our laboratory, demonstrating its reproducibility [manuscript in progress]. [20,76] In rodents, the ligation of both uterine arteries near the bifurcation prevents contamination of blood flow to the un-ligated horn. In this study, the insult was given near the end of gestation (GD20) as it reflects a similar stage of vulnerability to the third trimester of human pregnancy. [18,71] Overall, this model has previously been shown to produce IUGR offspring with subtle, diffuse brain injury and robust behavioural, cognitive, and motor impairments consistent with a CP phenotype [manuscript in progress]. [20,76] This subtle global injury has also been described by Arthurs et al., who found abnormal maturation in white matter and thalami of IUGR fetuses compared to controls despite normal gross brain morphology, and Olivier et al., who found diffuse white matter damage, macrophage infiltration and astrogliosis in IUGR offspring from unilateral artery ligation. [236,247] Generally, large behavioural abnormalities are accompanied by gross pathological changes. Therefore, investigations into neuronal morphology were warranted to determine the role the neuron plays in the pathology of IUGR.

In this study, 200mg of BrSp was supplemented to the dam daily. This dose has been shown to confer prevention of pathological and behavioural abnormalities in newborn and adult IUGR offspring of this model [manuscript in progress].[20,76] This dose has also shown to improve newborn reflexes of offspring in a model of *in utero* inflammation.[228] Notably, this amount of BrSp has been shown to provide the equivalent of 500µg of SFN to the fetus, demonstrating the ability of maternally consumed BrSp to effectively provide SFN to the fetus across the placental barrier.[76]

Rats, specifically Long-Evans, were selected for their large litter sizes, short gestational period, cost effectiveness, and similar placental and brain structure to humans.[71,73] The altricial nature of rats allows for the reflection of developmentally equivalent premature infants (23-32 weeks gestation), which have the highest risk of NDD.[71] Moreover, this characteristic allows for the representation of a compromised in utero environment, making rats the ideal specimen. P35 was selected as the endpoint in this study for several reasons, 1) most neural network development and pruning has been completed by this age, 2) P35 has been determined to represent 11-13year old children, based on brain development milestones, a time by which the majority of NDD are diagnosed, and 3) this time point correlates to the start of behavioural testing in our previous work on this model [manuscript in progress].[20,71]

Golgi staining is a widely used method for investigating the morphology of a neuron in its entirety, and is still considered as one of the best techniques to explore brain cytoarchitecture.[248,249] Cox developed the Golgi-Cox reaction in 1891, as a modification of the original method by mixing mercuric chloride and potassium dichromate solutions with potassium chromate.[250] The

formation of a mercuric chromate precipitate inside the cell allows visualization of the neuron structure in great detail after further processing. [250] In this study the Golgi-Cox method was employed, as it is known for its lower background, utilization in animals of all ages, and for being the most reliable. [248,249,251] Moreover, only 1-10% of the neurons in the brain are stained at random, making it possible to visualize neuronal morphology virtually unobstructed. [248,250]

8.2 Weights and Viability

Offspring in this model were born with varying degrees of asymmetrical IUGR, which has been shown to depend on their location in utero. [74] To answer our research question, whether or not BrSp would prevent IUGR brain abnormalities, only offspring who met the criteria of IUGR were included from BUAL dams. Therefore, Sham animals weighed significantly more than IUGR offspring across the time points. As expected, offspring mortality is high following BUAL and BrSp did not prevent IUGR from occurring. These results can be explained by the nature of the model used as well as our inclusion criteria. The BUAL model relies on mechanical disruption of blood flow through ligation of the uterine arteries. This interruption in blood flow thereby prevents any beneficial effects of BrSp on vasodilation, although the effectiveness of anti-inflammatories and anti-oxidants in preventing vascular dysfunction has been shown in several studies and varying arteries. [20-22] As mentioned above, OS and inflammation can disrupt endothelial function further increasing OS in a cyclical manner. [66,89] Breaking that cycle with an antioxidant and anti-inflammatories, such as BrSp, would not be of benefit for the prevention of endothelial dysfunction in this model. Conversely, in a model of biological disruption such as maternal lipopolysaccharide injections, as seen by Nguyen et al., BrSp demonstrated the ability to prevent dysfunction, allowing normal fetal growth to occur. [228] Nguyen et al. also showed

increased offspring viability with the supplementation of BrSp into the maternal diet, bolstering this hypothesis. [228]

Although IUGR offspring did not fully display catch up growth at our endpoint (P35) they did display rapid growth. Moreover, it has been previously shown in this model that by P80 IUGR weights caught up to controls [manuscript in progress]. [20] This exponential growth has been displayed in children with fetal growth restriction due to a higher fat mass to skeletal muscle ratio, placing them at risk for later development of obesity and cardiovascular diseases. [252-254] Although body composition was not evaluated in this study, it can be speculated that the rapid growth of IUGR offspring may be attributed to fat mass accumulation. Alternatively, BUAL dams may have adapting milk content to compensate for small offspring weights thereby enabling faster growth of the offspring. [255] In this regard, a systematic review revealed that preterm breast milk has higher protein content than term milk, which may impact growth of the infant. [256] Furthermore, IUGR animals could have an increased appetite post-weaning, which could account for the rapid weight gain. Future investigations will be needed to confirm these hypotheses.

8.3 Cortical Thickness

Surprisingly, no significant differences in cortical thickness were found between the groups. Typically, cortical thickness is used as a rough morphometric measure of cell number as the development and migration of cells causes cortical thickening during development. [245] Therefore, the thickness of the cortex reflects the density of neurons and glia. In this regard, decreased cortical thickness is associated with increased atrophy, which in turn is associated with

decreased connectivity and altered neuronal morphology.[245] Narr et al. showed that cortical thickness correlated to gray matter concentrations, calculated based on signal intensity of magnetic resonance images, in schizophrenia patients.[245] Gray matter contains neurons, dendritic trees, and spines, the functional units of the brain that integrate and transmit information. Thus alterations in gray matter may reflect changes in neuronal morphology and function of the brain. To this end the correlation of cortical thickness and intelligence has been shown in several studies.[257-259] In this study, no differences were found in cortical thickness measurements, indicating that the overall cell number in the cortex is the same across groups. Contrary to our findings, Gressens et al. utilized a malnutrition model of IUGR in the rat, which showed changes in cortical thickness at GD15 compared to controls.[260] The differences in the severity and phenotype of IUGR between malnutrition studies and our PI model could explain the dissimilarities in cortical thickness measures. However, Gressens et al, also found that the changes in cortical thickness disappeared in later age suggesting recovery, which may also be the case in this study.[260] The developing brain has an incredible capacity for plasticity to injury compared to the adult brain.[261] In the rat cortex, proliferation of cells is occurring around the time of injury in this model (GD15-21) and dendritic growth peaks around P7-10.[261] Injury to the developing brain during this time period may activate compensatory neural progenitor cell activation and neurite growth, facilitating recovery. However, although structural recovery may be occurring, leading to normal cortical thickness measures, the function of the cells after injury may be altered.

Along with neuronal loss, the pathology of cortical thinning includes cortical demyelination and degeneration from white matter lesions. The insult in this study is inflicted

during a time when preOLs, a cell exceedingly vulnerable to oxidative injury, dominate. [34]As mentioned previously, injury to these cells can result in cell death, loss of neuronal-OL communication, and axonal degeneration. [203]Axonal degeneration impacts the overall function of the neuron and dendritic morphology as decreased synaptic activity would result in dendritic degradation. [145,203,204] Notably, Back et al. has shown that progenitor cells replace preOLs after death. [18,33,34] Unfortunately, these progenitor cells are unable to mature to produce myelin. [18,33,34] Thus, a recovery in immature OL cell number may occur, lending to the lack of cortical thickness changes seen in this study, although with altered function that nevertheless impacts neuronal morphology.

Lastly, these disparities may be due to the methodology used. In our study, measurements were not done stereotactically and were taken from one hemisphere at random because of the global diffuse nature of injury inflicted by this model. The nature of this method may not provide an accurate representation of the overall thickness of the cortex, preventing the detection of differences in this study. This is evident in the results from sophisticated magnetic resonance imaging (MRI) studies. Dubois et al. evaluated preterm IUGR infants using MRI and found thinner cortices as well as decreased cortical volume compared to controls. [121] Similarly, Egana-Ugrinovic et al. found decreased cortical thickness of the insula in growth-restricted infants using MRI. [262] The sensitivity of MRI may explain the ability to detect differences in newborn brains in these studies. Therefore, the utilization of more sensitive techniques such as MRI may provide more reliable results. Alternatively, the subtle and diffuse pattern of injury displayed in this model may lead to non-detectable differences in cortical thickness, no matter the method of assessment, as the injury may not be extensive enough to cause such changes.

8.4 CA1 Morphology

Basilar morphological measurements in the CA1 region showed neuropreventive benefits of BrSp supplementation. Basilar dendritic retraction and fewer total BPs were observed in IUGR offspring compared to both control and IUGR+BrSp groups. In line with this, Dieni and Rees found that PI induced IUGR guinea pig offspring had decreased DL in CA1 basilar dendrites.[235] Notably, the CA1 region of the hippocampus has been shown to be particularly vulnerable to hypoxic-ischemic insults and OS.[240,263] Excessive glutamate release due to ischemia/OS can alter dendritic growth of these hippocampal neurons as it has been shown to suppress growth cone activity.[264] Additionally, OS can cause Ca^{2+} influx, which has been shown to alter dendritic growth.[265] Sustained increases in intracellular Ca^{2+} as a result of glutamate exposure, as seen in chronic OS, can cause abnormal microtubule formation in dendrites leading to retraction.[266] Moreover, we found that IUGR+BrSp offspring did not differ from controls in basilar DL or complexity measures. These findings complement our previous data, in that IUGR adult offspring of this model had deficits in spatial learning strategy and high anxiety, which were prevented with BrSp supplementation [manuscript in progress], as the hippocampus is universally known to be involved in learning and memory.[20,263] In addition, our laboratory has shown decreased neuronal cell counts in the CA1 region for P21 IUGR offspring of this model.[76] It was also shown that maternal BrSp supplementation partially prevented this cell loss due to IUGR, further complementing the findings of this study.[76]

As predicted, BrSp supplementation during gestation was able to prevent some structural alterations of CA1 neurons caused by PI induced IUGR. As previously discussed, the consumption of BrSp metabolically produces SFN, which induces the transcription of

antioxidant and detoxification enzymes through the mediation of the Nrf2 pathway (Figure 3). [230,231] The transcription of several enzymes involved in GSH synthesis and GSH regeneration through this pathway increase the ability to detoxify ROS caused by this model. [100] GSH deficiency has been shown to cause mitochondrial damage in the brain and is implicated in many neurodegenerative and neurological disorders. [267,268] Moreover GSH has been shown to be significantly lower in IUGR brains. [269,270] Increasing the production and function of this essential antioxidant, through BrSp consumption could therefore prevent OS and damage. Additionally, BrSp contains vitamins, Cys, polyphenols, and folic acid, among other natural bioactive substrates, that can combat oxidative stress and inflammation.

8.5 The Role of Astrocytes in BrSp Neuroprevention

Due to the intimate communications between neurons and glia, the fact that glia outnumber neurons, and the role astrocytes play in homeostatic functions to reestablish neuronal integrity, any injury to the brain likely affects astrocytes. Thus, understanding their role in this model could provide a better grasp of the mechanisms by which BrSp affords protection. Astrocytes play a key role in neuronal survival through GSH metabolism and therefore may be the primary target of the beneficial properties of BrSp. [103,267,271,272] Astrocyte endfeet surround blood vessels in the brain making them essential to the blood brain barrier, beneficially the first in contact with energy substrates and amino acids delivered to the brain, and consequently the first to encounter toxic compounds. [103,110,185,273] Accordingly, astrocytes are the first line of defense for the brain and must therefore be equipped with abundant detoxification mechanisms. GSH synthesis is one such mechanism. Astrocytes have a high cellular GSH content as well as a large efficient capacity for GSH detoxification and synthesis that not only benefits astrocytes but

surrounding neurons. [100,108-113] Essential to GSH production is the availability of amino acid substrates and activity of the enzymes involved in synthesis (Figure 2). [100,110] In this regard, Sagara et al. showed that neurons could not maintain Cys levels without astrocytes. [112] Cys is a rate-limiting building block of GSH synthesis, which is rapidly oxidized into cystine extracellularly. [100,112] Sagara et al. also demonstrated that neurons were only able to utilize Cys in GSH synthesis, while astrocytes could use both Cys and cystine in culture, demonstrating the vital importance of astrocytes ability to convert cystine to Cys in neuron GSH synthesis. [112] Once converted, Cys is secreted by astrocytes for neuronal uptake thereby contributing to the maintenance of GSH levels in neurons. [110]

Although the direct application of GSH precursors increases GSH levels, the availability of glutamate-cysteine ligase, the rate-limiting enzyme essential for combining Cys and glutamate, dictates GSH synthesis. [112,274] Thus, inhibiting glutamate-cysteine ligase decreases brain GSH content. [275,276] The transcription of glutamate-cysteine ligase and glutathione synthetase is controlled by activation of the ARE, which is in turn regulated by Nrf2. [110,231,277] As discussed above, SFN provided by BrSp activate the Nrf2 pathway, thereby increasing the production of these enzymes (Figure 3). [230,231] In addition, astrocytes have been shown to be the preferential target for Nrf2 pathway activation under OS. [103-105,278-280] Shih et al, found that astrocyte enriched cultures expressed ~12 fold more Nrf2 protein than neuron only cultures, implying higher antioxidant gene expression in astrocytes. [278] Consequently, disturbed astrocytes, such as in OS, may significantly contribute to abnormal neuron differentiation and altered arborization due to the critical roles they play in antioxidant systems as well as neurite growth and dendrite functioning. Therefore, increasing the availability of Cys and GSH enzymes

through BrSp supplementation can theoretically increase GSH levels in the brain, and may be a contributing factor to the neuroprevention BrSp affords. [274] Overall, BrSp may be activating the Nrf2 pathway through astrocytes, providing ample Cys to facilitate GSH synthesis, and ultimately affording neuroprevention. This hypothesis is supported by previous results in our laboratory that found significantly more reactive astrocytes in both the corpus callosum and cingulum of IUGR offspring of this model. [76] IUGR+BrSp offspring were also shown to have significantly fewer reactive astrocytes as compared to IUGR only offspring, demonstrating the increased capacity of astrocytes to combat OS and inflammation with supplementation. [76] Therefore, focusing on neurons alone would be insufficient in understanding the role BrSp plays in improving outcomes.

8.6 CA1 Spine Density

Spine density measures were investigated, as spines are the location of neural communication via synaptic transmission. [160] Moreover, spine density has been associated with neurological disorders and NDD. [160,167,281-283] In this study the only significant differences in spine density were found on the basilar dendrites in the CA1 region. The establishment of appropriate dendritic spine density is influenced by several factors, such as N-methyl-D-aspartate-mediated synaptic activity and neurotrophic factor levels. [160,284-286] As described previously, the ischemic insult and OS caused by PI in this model can cause excitotoxicity, which can result in compromised synaptic pruning and altered synaptic transmission ultimately decreasing spine number. [68,94,287] Therefore, the decreased spine density found in the basilar CA1 neurons of IUGR offspring may correlate with the loss of synaptic terminals, which would remain permanent if regrowth of axons does not occur. [288] To this end, Cheng et al. demonstrated

spine density recovery after cortical lesions that were induced by axonal sprouting after injury.[289] The decrease in DL and complexity found in the basilar CA1 for IUGR offspring therefore could explain the concurrent decrease in spine density. Furthermore, altered synaptic innervation through decreased spine density could result in morphological changes in DL and complexity, therefore proposing a 'chicken versus egg' scenario.[37] Zhao et al. also found decreased spine density in the CA1 region of animals exposed to a hypoxia-ischemia insult through carotid artery ligation, supporting these results.[290] No significant differences were found for apical CA1 spine density measures in this study. In contrast to these results, Dieni and Rees found significant increases in spine density in CA1 apical dendrites of growth restricted guinea pig offspring, compared to controls.[235] The use of a precocial animal by Dieni and Rees, as well as the analysis of second order dendrites instead of terminal ends, may explain the difference in results from this study.

Interestingly, BrSp supplementation was also shown to decrease spine density in the basal CA1 dendrites, although DL and complexity were increased in IUGR+BrSp offspring. One possible explanation for these results is that BrSp supplementation prevented DL and complexity degradation in IUGR animals without an accompanying increase in spine density. This decreased spine density together with increased DL and complexity in the same area questions if an overall change in synapse number is occurring. Kolb et al. was posed with this same problem in juvenile rats exposed to complex housing from P22-120.[291] In that experiment, Kolb et al. estimated the overall synapse number by multiplying the spine density by the DL.[291] Unfortunately, this calculation could not be applied to this experiment as not all animals completed both dendrite and spine analysis due to staining inconsistences and fading. Moreover, the possibility that the

changes in basilar CA1 spine density caused by BrSp supplementation may have a detrimental effect on the function of these cells should not be ignored. Therefore, further investigation into the net change of synapse number due to BrSp supplementation and the function of these cells is warranted, although previous work conducted in this model did not find any detrimental effects of BrSp on behavioural outcomes [manuscript in progress].[20] Moreover, the morphology of the spines was not investigated in this study, which could have provided insight into whether or not BrSp supplemented groups had a larger number of morphologically mature spines alongside lower total spine density, implying functional compensation.

8.7 Apical Dendrites in Pyramidal Neurons

PNs are known for their bi-conical shape segregating the basal and apical domains. As discussed earlier, the morphological differences, distinct regions of input, and separation of these domains is thought to represent functional distinctions.[170] Moreover, apical dendrites have been shown to have weaker effects on action potential propagation and smaller protracted excitatory postsynaptic potentials.[173] Here, no significant differences of BrSp were found in DL or complexity for the apical dendrites of the CA1 region. Moreover, only male IUGR offspring were significantly affected in the apical CA1 complexity and length measures. These results may be explained by the functional differences between apical and basilar dendrites, as apical activation may have a modulatory role over the action potential driving basal inputs.[171] As apical activation is smaller and unreliably propagated, these dendrites may be more stable and less impacted by changes in the environment. Additionally, CA1 basilar dendrites receive input from neighbouring CA1 and CA3 neurons, which have the highest vulnerability to hypoxic conditions and oxidative stress, while apical dendrites of this region receive inputs in a top down manner

from the entorhinal cortex. [170] This separation of information input and functional distinctions may explain the lack of morphological disruption in the apical dendrites.

8.8 M1 Morphology

Basilar M1 neurons also displayed an effect of BrSp, however no abnormalities were detected in IUGR offspring. BrSp were shown to increase DL and complexity regardless of surgery, indicating the importance of a healthy diet during pregnancy. Although more does not always mean better, previous work done with this model in our laboratory did not show any detrimental effects of BrSp supplementation on the behaviour or pathology of Sham offspring [manuscript in progress]. [20,76] Therefore, we can assume that the observed increases in basilar DL and complexity in the primary motor cortex are due to an improved maternal diet, and are not detrimental. Alternative explanations for the lack of observed injury in IUGR offspring could be that, 1) the injury may impact more superficial layers of the cortex, such as layer III, more substantially than layer V, and 2) that given the age of these animals, some recovery may have occurred in this region explaining the lack of damage. Moreover, unless sophisticated brain imaging techniques are used, cortical neuron involvement is uncommon in preterm ischemic infants. [262,292,293] This may be due to the immaturity of the cortical neurons at the time of injury. [261] This does not, however, rule out the possibility of neural changes in other motor regions of the brain such as the basal ganglia.

8.9 The Effect of Sex

Sex differences were also found in this experiment. Overall, male offspring were predominately affected by IUGR in this model. In the CA1 region male IUGR offspring had shorter basilar and apical dendrites, as well as significantly less apical BPs compared to both IUGR females and Sham males. These results complement the human literature in that males have a higher incidence of NDD compared to females. [1] Moreover, it is well known that reproductive hormones impact several aspects of neurodevelopment and brain functioning, including neuronal morphology. [294,295] In this regard, Juraska et al. has shown sex differences in DL in the CA3, with females having more dendritic intersection in the proximal apical tree and males having more in the distal apical tree. [296] Previous work in our laboratory also demonstrated sexual dimorphism in behavioural, cognitive, and motor impairments of this model, with males overall showing more detrimental outcomes [manuscript in progress]. [20]

8.10 Conclusions

BUAL induced PI resulted in IUGR offspring and increased fetal mortality, regardless of BrSp supplementation. Although BrSp supplementation did not prevent IUGR from occurring, Golgi-Cox staining of brain tissue revealed neuropreventive benefits, specifically in the basilar CA1 dendrites. Alterations in both basal DL and dendritic complexity caused by IUGR were prevented following maternal BrSp consumption during pregnancy and lactation. IUGR did not adversely affect neurons in layer V of the M1 or overall cortical thickness at the time point evaluated, therefore BrSp effects on IUGR were undetectable. Interpretation of the effects of BrSp on our current spine density results is challenging, as the overall net change in synapses

could not be calculated and staining difficulties may have contributed to the differences found. Nonetheless, the neuropreventive effects BrSp affords as a novel therapeutic intervention for *in utero* insults such as IUGR is evident. Alterations in the structure of neurons strongly suggest functional abnormalities. [131,177,178] In this regard, disruptions in neuronal maturation demonstrated in the CA1 can, in part, explain the spatial learning abnormalities seen in our previous long-term behavioural work [manuscript in progress]. [20]

Our model of PI represents the majority of IUGR cases in the developed world. [47] Furthermore, IUGR has been strongly associated with the development of NDD; including a 10-30 fold increased risk of CP. [9,47] Preventative therapies that target antepartum insults, such as IUGR, and therefore the majority of children that ultimately develop NDD, are currently lacking. [7] Therefore, the development of a safe and efficacious neuropreventive intervention that can potentially target infants at risk of NDD and be safely administered during gestation, is critical. BrSp could be the answer. Several studies including those from our laboratory have provided evidence for the safety and efficacy of BrSp supplementation during pregnancy [manuscript in progress]. [20,76,226-228] The results from this study demonstrate that adverse *in utero* conditions can impact dendritic maturation, with varying effects across region. Moreover, maternal BrSp consumption during pregnancy and lactation can impact dendrites throughout the brain. Therefore, this study highlights the use of BrSp dietary supplementation as a promising neuropreventive intervention during pregnancy that prevents alterations in neuronal morphology caused by IUGR, and is implicated in several NDD. These results are encouraging for the eventual reduction of devastating NDD, such as CP.

9 LIMITATIONS

The time consuming process of the Golgi-Cox technique prevented the exploration of several brain regions of interest, including layer II/III of the M1, the striatum, and prefrontal cortex. Alongside these regions, investigating varying time points during development could provide insight into the temporal changes caused by IUGR and the effects of BrSp.

Limitations of this study relate to the lack of maternal investigations, interpretation of morphological impact on neuronal function, and the probability of human error due to staining variations. Dams that underwent BUAL surgery were notably in more discomfort and exhibited greater lethargy than those that underwent sham operations. The difficult recovery from BUAL surgery may have impacted maternal food consumption, which, as previously mentioned, can result in IUGR. Malnutrition and dietary changes before or during pregnancy can change maternal protein and fat deposits, which can alter the composition of milk produced. [255,297-300] Gressens et al. showed that maternal protein restriction caused abnormal synaptogenesis and neuronal differentiation in offspring. [260] These negative effects on brain development can ultimately impact offspring behaviour and cognition. [301] Investigations into maternal food and water intake after surgery and the impact on milk composition missing from this study may have shed light on the effects of maternal nutrition on offspring outcomes. These alterations may have further potentiated the damaged caused by BUAL, and must be considered a cofounding variable. Additionally, maternal stress and anxiety during pregnancy, such as that of surgery, alters postpartum behaviour and offspring care. [302-305] Furthermore, inflammation cause by abdominal opening during surgery can lead to changes in maternal behaviour. [306,307] Smit-Rigter et al. found that maternal licking and grooming behaviours altered dendritic morphology and

synaptic function in cortical pyramidal neurons.[308] Similarly, maternal care has been shown to increase synaptic density and neuronal survival in the hippocampus, as well as increased density in the medial prefrontal cortex.[309-311] Kolb and Gibb showed that tactile stimulation, normally provided by the dam, improves recovery from cortical injury to the developing brain measured through behavioural and neuronal morphological measures.[312] Therefore, a potential limitation of this study is the lack of cross-fostering of offspring due to nature of BrSp supplementation. Ultimately these maternal alterations can significantly impact brain development, imposing another confounding factor. Evaluating the effects of the dam in this model could provide insight on the overall maternal influence on offspring outcomes. However, increasing the dam number per group in this study was done to potentially circumvent these confounds.

In addition, this study is limited in the ability to interpret functional associations as a result of morphological changes. Neither behavioural nor electrophysiological assessments were completed in this study, therefore preventing the correlation of morphological alterations with functional outcomes. However, the structural changes of neurons have been shown in several neurological disorders and correlated to function.[156,167,283,290,313] Moreover, alterations in dendritic complexity and length have been shown to impact the function of the neuron in terms of action potential firing and patterns.[177,178] Furthermore, behavioural abnormalities have been demonstrated in animals from this model suggesting an association between morphology and function [manuscript in progress].[20,76] In addition, analyses were limited to P35, preventing the ability to determine temporal changes in neuronal morphology and the effects of BrSp at earlier time points.

Lastly, there was limited consistency of tissue staining across animals. The difficult and inconsistent nature of Golgi staining is well known, and has been under constant modification since its inception to increase reproducibility, uniformity, and reduce processing time. This is evident in the many different types of Golgi protocols among variations in incubating temperatures and microwave energy treatment. [248,249,314-316] Moreover, Golgi-Cox staining is known to fade, which was seen in our tissue a year after processing. These problems may have led to error in tracing and analysis. Furthermore, tracing using the camera lucida is a time consuming, difficult, and subjective method, which increases the probability of human error. Strict criteria and one experimenter throughout the study were implemented to reduce this subjectivity and error.

10 SUMMARY

In summary, the model used in these studies mimics the prevalent human condition of PI, resulting in an injury during fetal development that ultimately leads to IUGR and morphological alterations of the neuron. These detrimental changes were prevented with the use of BrSp as a therapeutic intervention during the last trimester of pregnancy and lactation. As IUGR, and structural changes to the neuron are associated with NDD, identifying safe and effective interventions is crucial to reducing the incidence and severity of such disorders. BrSp afforded neuroprevention without any apparent adverse outcomes, therefore its use, as a novel therapeutic intervention against IUGR and NDD is promising. As more infants from adverse *in utero* environments survive, due to medical advancements, developing preventive treatments is crucial for the health and quality of life of our future children.

11 FUTURE DIRECTIONS

Future investigation in this model using electrophysiology could provide insights into the effects of IUGR and BrSp supplementation on neuronal function. The patch clamp recordings would be a beneficial avenue to explore, as it would provide insight into the overall electrical properties of cells and thus their information processing abilities. The characterization of activity differences between axons and dendrites, or between apical and basal dendrites could also be obtained, adding functional interpretation to results of this study.

Investigating NT release in this model could further provide evidence for the impact of IUGR and the effects BrSp supplementation on neurons, as NTs play an important role in neurite growth, neuron morphology, and synaptogenesis. [317-320] Furthermore, NT and neurochemical concentrations are altered in uterine artery ligation models performed on both guinea pigs and rats. [269,321] Vazquez-Gomez et al. found significantly higher concentrations of 3,4-dihydroxyphenylacetic acid and homovanillic acid, NT metabolites, in the hippocampus and amygdala of IUGR piglets. [322] Moreover, following uterine artery occlusion, Hernandez-Andrade et al. found decreased striatal GABA as well as increased glutamate and dopamine in the cortex, striatum, and hippocampus of IUGR rabbit kits. [155] Even more interestingly, SFN has been shown to impact dopamine and metabolite levels. [323] Therefore, looking at NTs in our model of IUGR and if BrSp can afford neuroprevention is necessary.

Exploration of the impact of IUGR and BrSp on spine morphology would be an interesting study as a reduction of mature mushroom spines would decrease postsynaptic density and likely alter the function of the cell. [160] Mahmmoud et al, linked mushroom spines to memory

formation in radial arm maze trained animals. [156] Therefore, understanding how PI induced IUGR and BrSp change spine morphology could imply behavioural associations.

This study is limited in that it did not examine the effects of BrSp on glia, specifically astrocytes. As mentioned above, astrocytes are not only support cells of the neuron but activate the Nrf2 pathway, which is the hypothesized mechanism by which BrSp exerts neuroprevention. Future research assessing the interactions of neurons and astrocytes in this model would be of interest to further elucidate the mechanisms of BrSp, and determine if the neuroprevention afforded by BrSp supplementation is due to fortified astrocyte antioxidant systems and ability to reduce OS and inflammation. An additional avenue for exploration is glia function and integrity, such as astrogliosis, microglial activation, demyelination, as well as functional efficacy and orientation of white matter tracts. These investigations could also provide further insight into neuron-glial interactions and their relation to this model. It is possible that the effects of BrSp are not directly acting on the neuron itself, but rather are mediated through the protection of glial function and prevention of injury. Moreover, studying these aspects of glial function in this model could further verify the hypothesis that astrocytes are targeted by BrSp properties, which beneficially impacts neuronal morphology. Overall, the evaluation of the effects of BrSp supplementation on glia in relation to neurons requires further investigation.

In summary, the goal of the work completed here and these future studies is to ultimately determine the extent of the neuropreventive properties of BrSp supplementation as a novel therapeutic intervention that targets antepartum risk factors of NDD. This promising research

could reduce the incidence and severity of NDD caused by IUGR in a safe manner for both mother and fetus.

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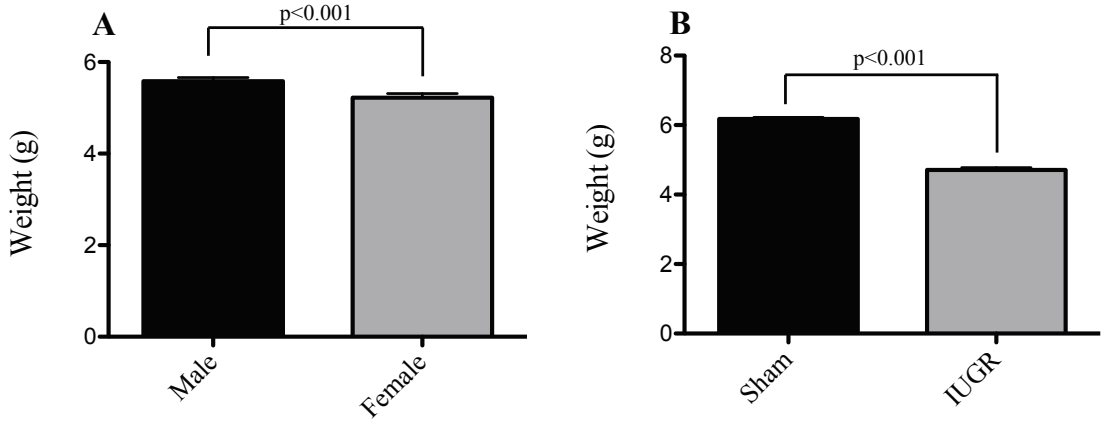
APPENDIX



Supplementary Figure 1: Birth Weights of all Offspring Regardless of Inclusion Criteria

The birth weights of all male and female offspring born to all dams in this study were recorded, regardless of inclusion criteria. Due to the necessity of equal numbers of each sex meeting inclusion criteria to reach statistical power in this study several litters were collected leading to varying numbers per group (Sham n=78, Sham+BrSp n=64, IUGR n=58, IUGR+BrSp n=137). Significant main effects of sex (A) (Male n=152, Female n=140) and surgery (B) (Sham n=108, IUGR n=195) were found. Data expressed as mean ± SEM.