Investigating the Role of *In Utero* Inflammation on Neurodevelopment in the Fetus and Offspring and the Neuroprotective Properties Afforded by Broccoli Sprouts

by

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ABSTRACT

Introduction

Maternal infection and inflammation leading to a fetal inflammatory response (FIR) is a risk factor for perinatal brain damage. Perinatal brain injury can lead to neurodevelopmental disorders, the *sine quo non* outcome parameter of which is cerebral palsy (CP). Despite advances in maternal and fetal medicine, no preventive therapy exists to deter *in utero* inflammation and subsequent development of CP. The objectives of this thesis are to: verify a model of fetal inflammation leading to phenotypic features of CP; identify the mechanisms involved in fetal inflammation leading to this phenotype, and; determine if consumption of broccoli sprouts (BrSp) can prevent the abnormalities induced by this model of inflammation.

Methods

Pregnant Long-Evans rats were injected with lipopolysaccharide (LPS, 200 μg/kg) on embryonic days (E)19 and 20 every 12 hours. Beginning on E14, dams were randomly divided to receive BrSp dietary supplementation in addition to their regular chow, or not. Dams (and offspring) were divided into four groups: 1) Saline (control), 2) Saline + BrSp, 3) LPS, and 4) LPS + BrSp. Pups born underwent a battery of neurodevelopmental reflex and behavioural testing from postnatal day (PD)3-PD21. Placentas and fetuses were isolated on E19 and E22 to undergo cytokine and neurotrophic factor analyses. Uterine and umbilical arteries were imaged on E21 and excised on E22 to analyze blood flow and artery function. Pup's brains were harvested on PD1, 7, and 21 to undergo histological analyses.

Results

LPS pups born were growth restricted and smaller compared to Saline, Saline + BrSp, and LPS + BrSp pups. LPS pups were significantly delayed in several neurodevelopmental reflexes testing including hindlimb placing, cliff avoidance, and gait. LPS + BrSp pups performances on these reflexes were not different from controls. Furthermore, LPS and LPS + BrSp pups ambulated less following open field analyses. On PD21, a reduction in myelination was observed in the LPS and LPS + BrSp groups compared to Saline and Saline + BrSp. To confirm the model elicits a FIR, placentas and fetal brains were examined for changes to cytokine expression on E19 and E22. On E19, an increase in interleukin (IL)-1β, TNF-α, IL-6, and IL-10 was detected in the LPS and LPS + BrSp placentas. In the fetal brain, a significant increase in pro-IL-1β was detected on E22 in the LPS group compared to Saline, Saline + BrSp and LPS + BrSp groups. On PD1, the ratio of pro-inflammatory cytokines protein levels, normalized to IL-10, was analyzed. A reduction in the TNF-α/IL-10 and IL-6/IL-10 was identified in the brains of females in both LPS and LPS+ BrSp groups. Analyses of neurotrophic factor expression, a possible downstream target of the cytokines, revealed a significant decrease in nerve growth factor mRNA production in the fetal brains at E22 in the LPS and LPS + BrSp groups. A significant reduction in 2',3'-cyclic-nucleotide 3'-phosphodiesterase, a marker of differentiated oligodendrocytes, was observed in the white matter of PD21 LPS pups. No other differences in maturation markers, utero- and umbilical-placental blood flow, and uterine artery vascular contractile and relaxation function were detected.

Conclusions

LPS induced maternal inflammation was sufficient to produce reflex and behavioural abnormalities in their offspring, with an early phenotype that is consistent with that of developmental disability and CP. An increased placental and fetal inflammatory response was observed along with a reduction in NGF production and 2',3'-cyclic-nucleotide 3'-phosphodiesterase in LPS pups. BrSp dietary supplementation significantly prevented growth restriction and some developmental reflex delays, the upregulation in IL-1β mRNA, and 2',3'-cyclic-nucleotide 3'-phosphodiesterase levels. The findings suggest that BrSp dietary supplementation during pregnancy is a novel, safe and efficacious preventive strategy in the challenge of treating cerebral palsy and the developmental disabilities.

PREFACE

The original research presented in this thesis was performed by Antoinette Nguyen. The animal work involved received ethics approval by the Animal Care and Use Committee, University of Alberta, AUP 00000364, Dr. Jerome Y. Yager, Principal Investigator.

The first objective of this thesis in regards to developmental reflexes, behaviour analyses, and white matter injury was published as Nguyen AT, Bahry AMA, Shen KQ, Armstrong EA, Yager JY. Consumption of broccoli sprouts during late gestation and lactation confers protection against developmental delay induced by maternal inflammation. *Behav Brain Res*. 2016;307:239-249. I had conducted, or had assisted in, all experimental procedures, and analyses in this manuscript, except for sectioning the brain tissue for histological analyses. KQ.S. sectioned the brains for subsequent histological analyses as well as helped with immunohistochemical staining. A.M.A.B. assisted in behaviour and histological data analyses. E.A.A. contributed to development of the project, helped breed the animals, and assisted in the reflex and behavioural aspects of the research. J.Y.Y was the principal investigator and established the research project as well as manuscript development.

DEDICATION

To my family, life partner, and friends, for their constant support and encouragement throughout my studies.

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LIST OF ABBREVIATIONS

BDNF – Brain Derived Neurotrophic Factor

BrSp – Broccoli Sprouts

CP – Cerebral Palsy

E – Embryonic Day

E_{max} – Efficacy (maximum vasoconstriction or maximum vasodilation)

FIR – Fetal Inflammatory Response

IFN – Interferon

IL – Interleukin

L-NAME - L-N^G-Nitroarginine methyl ester

LPS – Lipopolysaccharide

MMP – Matrix metalloproteinase

mRNA – Messenger RNA

NF-κB – Nuclear Factor kappa-Light-Chain-Enhancer of Activated B Cells

NGF – Nerve Growth Factor

NO – Nitric oxide

Nrf2 – Nuclear Factor (erythroid-derived 2)-like

NT-3 – Neurotrophin 3

PD – Postnatal day

pEC_{50} – Potency

pegSOD – polyethylene glycol superoxide dismutase

SFN – Sulforaphane

TGF – Transforming Growth Factor

TNF – Tumor necrosis factor

1. INTRODUCTION

1.1. PERINATAL BRAIN INJURY

Perinatal brain injury arises from insults that can occur at any time during gestation or in the newborn period, and leads to neurodevelopmental disorders such as cerebral palsy (CP), and mortality. These disorders cause a heavy socioeconomic and healthcare burden, as well as caregiver stress. Significant advancements in maternal and neonatal care have increased the survival rate of preterm infants to upwards of 90%, yet these infants are at high risk for morbidity. [1,2] It is estimated that CP will occur in 10% of preterm infants, and neurological disabilities such as seizures and intellectual disabilities will appear in another 25-50%. [2] Newborn brain injury occurs in 1-6/1000 live births. [3,4] Epidemiological studies have identified that the majority of risk factors such as infection and inflammation occur in the antepartum period (70-95%), and the minority occur during the intrapartum and/or postpartum period (5-30%). [1,3,5] The antepartum period represents a highly vulnerable time, during which the fetal brain must be protected. [6] The brain of the developing fetus is considered a 'moving target', since the patterns of brain injury associated with term infants involve the cortex and deep gray matter structures, whereas in the preterm infants, it involves the periventricular white matter tract and parenchyma. [7-9] Currently, the only neonatal treatment is hypothermia, a form of rescue therapy given within 6 hours of birth to infants exposed to intrapartum hypoxia. [10,11] Unfortunately, no intervention exists to prevent the majority of insults, especially those that occur during the antepartum period. This emphasizes a need to investigate therapeutic approaches that might protect the fetal or newborn brain.

1.2. CEREBRAL PALSY

1.2.1. Cerebral Palsy

CP is a non-progressive disorder characterized predominantly by motor deficits and associated additional co-morbidities such as intellectual disability and seizures. [12] CP is defined as a *"group of permanent disorders of the development of movement and posture, causing activity limitations, attributed to non-progressive disturbances that occurred in the developing fetal or infant brain…often accompanied by disturbances of sensation, perception, cognition, communication, and behaviour - epilepsy and musculoskeletal problems"*. [13] CP is a debilitating disorder, with a heavy burden on the affected individual and their caregiver(s). [14-17] The lifetime cost for one infant with CP is estimated to be 1.5 million US dollars. [1,18-20]

Despite advances in obstetrical and neonatal care, the incidence of CP has remained steady at 2-2.5/1000 term births, [1] and the ratio of males to females affected with CP is 1.4:1. [21] Wu et al. and Neufeld M et al. found that chorioamnionitis increased the incidence of CP and periventricular leukomalacia in both preterm and term infants by 2-4 fold. [22-24] Ancel et al. identified that children born at earlier gestational ages (24-26 gestational weeks) had a higher incidence of CP (20%) compared to those born later (after 32 weeks, 4%). [25] Oskoui et al. found the incidence of CP in a cohort of infants with a birth weight between 1000-1499 g was 59/1000, and in infants weighing <1000 g at birth, the incidence was 112/1000. [26] Neufeld et al. identified that the risk of CP escalates 20-fold if the fetus was exposed to *in utero* inflammation and born prematurely. [24] Moreover, the incidence of CP has not been declining, partially due to the

multitude of associated risk factors associated, which render the development of therapeutic interventions challenging.[27] This is a shortcoming that must be addressed.

1.2.2. Presentation

Not only are the risk factors associated with CP numerous, but patients with CP may not all present with the same imaging and physical characteristics. CP is categorized into three major topographic forms: spastic, dyskinetic, and ataxic, which can be further subdivided based on the afflicted anatomical areas. Spastic CP is the most prevalent (accounting for ~85% of cases) and results from damage to pyramidal neurons. Dyskinetic and ataxic CP, with prevalence rates of 8 and 5% respectively, arise from injury to extrapyramidal neurons.[28,29] The spastic form is characterized by muscle contractions and stiffness, and is more common in preterm infants.[28-30] Dyskinetic CP presents with involuntary movements and ataxia with inferior balance and coordination, and occurs more frequently in term infants.[30] Approximately 50% of individuals with CP also have co-morbidities including sensory impairments, intellectual disability, and neurodevelopmental disorders such as autism (6.9-18.4%) and epilepsy (41-67%).[29,31] Common cerebral abnormalities include periventricular leukomalacia and diffuse gray matter injury.[29,32] Towsley et al. found that the predominant forms of brain damage were periventricular leukomalacia and gray matter damage, present in 19% and 15% of the population respectively. Interestingly, 32% of individuals with CP present with nonspecific and/or absent findings, upon head computerized tomography and magnetic resonance imaging.[32] Together, the data convey the complexity of CP, a heterogeneous disorder of several differing etiologies.

1.2.3. White Matter Injury

Periventricular leukomalacia, a white matter injury, is the most common neuropathological feature associated with CP. [2] There are two forms of periventricular leukomalacia, cystic and diffuse, with the latter being predominant. Cystic periventricular leukomalacia is described as cysts within the brain parenchyma, formed by cellular necrosis. [2] Diffuse periventricular leukomalacia is an injury to a subset of cells, the oligodendrocytes, with diffuse activation of microglia and astrocytes. [2] Damage to the white matter has previously been ascribed as the primary brain pathology associated with CP, but this has now evolved to include gray matter damage. In support of this, Haynes et al. investigated immunomarkers of neuronal injury, β-amyloid precursor protein and fractin, in 17 control human brain tissue samples, and 13 samples with periventricular leukomalacia. [33] The β-amyloid precursor protein was detected in cystic periventricular leukomalacia and fractin was found in the diffuse form, confirming the presence of neuronal injury. [33] Pierson et al. analyzed 41 premature infants' brain tissue (control n=7; periventricular leukomalacia identified with focal necrosis n=17; and diffuse periventricular leukomalacia n=17) with hematoxylin and eosin and Luxol-fast-blue staining, and found neuronal loss localized primarily in the thalamus and globus pallidus. [34] This shows that the neuropathology of CP involves not only white matter damage, but can also effect neuronal maturation and survival. In summary, CP has a complex and heterogeneous pathophysiology, involving both white and gray matter. Ideally, a therapeutic intervention that targets a broad range of risk factors and pathological injury would be optimal.

Oligodendrocytes are a type of glial cell responsible for the production of myelin, commonly referred to as white matter, which insulate axons for proper nerve conduction and

provide growth factors for neurons. The oligodendrocyte lineage consists of oligodendrocyte progenitors (detected with the antigen A2B5+), preoligodendrocytes (O4+O1-), immature oligodendrocytes (O4-O1+), and mature oligodendrocytes (myelin basic protein).[35] At the O4+ stage, the oligodendrocytes begin to sheath the axons but are not capable of producing myelin until the mature stage. The preoligodendrocytes are the predominant cells surrounding the ventricular region between 24-32 weeks of gestation, corresponding to the period of highest vulnerability for developing periventricular leukomalacia and subsequently CP.[36,37] Preoligodendrocytes are vulnerable to oxidative stress, inflammation, and excitotoxicity, due to the underdeveloped vasculature, presence of calcium permeable glutamate receptors, localization of microglia to the white matter, and immature antioxidant system.[2,38,39] Ultimately, injury to pre-oligodendrocytes causes cell death and maturation blockade, resulting in periventricular leukomalacia.[38,40,41]

Preoligodendrocytes are vulnerable to oxidative stress due to the absence of a mature antioxidant system; they lack important enzymes such as manganese- and copper zinc containing superoxide dismutase, glutathione peroxidase, and catalase.[2,42,43] Oxidative stress results from an imbalance of pro-oxidants to anti-oxidants, where free radicals are not properly removed from the cell. The body normally produces pro-oxidants, such as superoxide anion, which is commonly produced by the mitochondria or enzymes involved with oxidation/reduction. Generation of superoxide anion involves the transfer of an electron (one electron reduction) to oxygen.[44] Superoxide anion can interact with superoxide dismutase, which facilitates the conversion of the free radical to hydrogen peroxide.[44] Hydrogen peroxide can either be broken down to water and oxygen by the enzyme catalase, converted to the hydroxyl radical by the Haber-Weiss reaction, or transformed into water by glutathione peroxidase.[44] The superoxide

anion can also react with nitric oxide (NO), forming the potent pro-oxidant, peroxynitrite. [44] In the presence of carbon dioxide, peroxynitrite is transformed into nitrosoperoxycarbonate, and with the addition of a proton, generates nitrogen dioxide radicals and carbonate. [44] In an acidic environment, peroxynitrite is protonated resulting in the formation of peroxynitrous acid, which can further generate hydroxyl and nitrogen dioxide radicals, as well as nitrite and sulphenic acid. [44] This complex system emphasizes the importance of having a functional antioxidant system to remove pro-oxidant by-products.

Folkerth et al. examined the parietal white matter in human brain tissues, in infants ranging from 18-214 weeks post conception (n=42). [45] The expression of both copper zinc and manganese superoxide dismutases was delayed, and was lower than adult levels present in the parietal white matter, whereas glutathione peroxidase and catalase expression exceeded adult levels by 30 weeks. [45] Baud O et al. cultured primary rat oligodendrocytes and induced oxidative stress by depleting cystine in the culture medium. [46] Cystine deprivation acutely reduced cell survival of pre-oligodendrocytes whereas mature oligodendrocytes maintained viable for longer. [46] The reduction in cystine was paralleled by an increase in superoxide generation. The resistance of mature oligodendrocytes to cell death induced by cystine deprivation was due to increased protein expression of manganese superoxide dismutase. [46] These studies emphasize that during the developmental window of susceptibility to periventricular leukomalacia, there is a low expression of antioxidant enzymes. Following maturation of oligodendrocytes, an upregulation of antioxidant enzymes conferred neuroprotection against oxidative stress-induced damage. The predominant cause of CP is injury to the pre-oligodendrocytes, which are susceptible to oxidative stress, primarily resulting from hypoxic-ischemic insult. However, inflammation is also

recognized, as a significant contributor to the etiology of CP. Therefore, understanding the mechanisms associated with inflammation-induced encephalopathy is important.

1.3. INFLAMMATION INDUCED ENCEPHALOPATHY

1.3.1. Inflammation Induced Encephalopathy

Newborns exposed to *in utero* inflammation are at increased risk of neurodevelopmental (i.e., CP) and neuropsychiatric disorders, and death. [36,47-51] Intrauterine infection leading to chorioamnionitis, a maternal inflammatory response, is more likely to occur in mothers of preterm infants than term infants, and is correlated with a subsequent risk for developing CP. [22,23] However, gestational period is not a protective factor once *in utero* inflammation is present and the risks for adverse neurodevelopmental outcomes is similar across all ages. [52] Moreover, activation of certain Toll-like receptors, a group of innate pathogen recognition receptors that recognize infection, can produce different patterns of cytokine expression, and lead to different disorders observed later in life. [53,54] Inflammation is a complex and dynamic process, which has led to investigation into the pathophysiolic mechanisms induced by maternal inflammation that lead to fetal brain injury and the diverse range of consequent disorders.

1.3.2. Maternal Inflammation

In utero inflammation can be of bacterial, viral, or parasitic origin. [55,56] Infection causes a maternal inflammatory response such as chorioamnionitis, and consequently, a fetal inflammatory response (FIR) that leads to neurodevelopmental disorders and preterm birth. [57-60]

Chorioamnionitis refers to inflammation of the placental membranes, the chorion and amnion, which normally protect the fetus from the external environment. [60-62] The routes of infection caused by microorganisms (i.e., *Ureaplasma* species and *Mycoplasma hominis)* during pregnancy leading to chorioamnionitis can be hematogenous, iatrogenic, or most commonly, ascending from the genital tract. [57-60] In the United States, chorioamnionitis is identified in 1-4% of all pregnancies, where 1-13% is identified in term births and 25-70% are associated with preterm birth. [27,60] There are two forms of chorioamnionitis, clinical and histological. [60] Common symptoms for diagnoses of clinical chorioamnionitis include fever, maternal and fetal tachycardia, foul discharge, and uterine tenderness. [60,62] Histological chorioamnionitis is diagnosed following histological abnormalities of the placental membranes, with leukocyte infiltration. [60] Upon severe chorioamnionitis, the inflammatory response can extend to the decidua (deciduitis), umbilical cord (funisitis), and placental villi (villitis). [60,62] Irrespective of the form of chorioamnionitis, the inflammatory response is associated with periventricular leukomalacia and a four-fold increase risk for developing CP. [22,23,63,64]. Wheater et al. also identified a four-fold greater risk of developing CP in infants who were born with low birth weights and had evidence of infection. [65]

The current treatment of chorioamnionitis is administration of antibiotics; however, classical antibiotics do not provide protection against *Ureaplasma*, a common microorganism identified in women with chorioamnionitis. [60] Typically, clinical chorioamnionitis is not identified until the patient presents with clinical symptoms, and histological chorioamnionitis is not diagnosed until after birth when the placenta can be examined for pathology. This suggests

that therapeutic interventions may not be given in time to protect the fetal brain from the inflammatory cascade that occurs following infection, leaving the brain vulnerable to injury.

1.3.3. The Fetal Inflammatory Response

Bacteria and/or maternal inflammatory mediators can degrade placental membranes and gain access to the fetus via the amniotic fluid. As mentioned, common pathogens found in chorioamnionitis are *Ureaplasma* species and *Mycoplasma hominis*, both of which are Gram-negative. [60] Gram-negative bacteria contain lipopolysaccharide (LPS) in the outer membrane, which is the endotoxin responsible for eliciting the immune response. Toll-like receptors are a type of pattern recognition receptor that detect motifs of microorganisms and activate the immune response to properly remove the foreign particles. [66] Currently, there are 11 known Toll-like receptors in humans and 13 in mice. [67] LPS is a ligand for Toll-like receptor-4, whereas Group B *Streptococcus* is a ligand for Toll-like receptor-2, and the viral mimetic polyinosinic:polycytidylic acid is a ligand for Toll-like receptor-3. [68-70]

Circulating LPS will adhere to two proteins: LPS binding protein and the cluster of differentiation 14, a co-receptor that recognizes LPS. This complex binds the Toll-like receptor-4, stimulating recruitment of several adaptor proteins. [66] The adaptor proteins will stimulate two possible pathways, the myeloid differentiation primary response gene 88 dependent pathway or the myeloid differentiation primary response gene 88-independent pathway. [66] The myeloid differentiation primary response gene 88 dependent pathway activates the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1. [66]

The myeloid differentiation primary response gene 88 independent pathway also stimulates activation of the transcription factors NF-κB, activator protein-1, and interferon (IFN) regulatory factor 3. [66] Toll-like receptor expression has been identified in several organs of the fetus, implying that the fetus may have the capacity to respond to pathogens and mount an endogenous inflammatory response. [71-75] Interestingly, Toll-like receptors are also found in the brain, and some have been suggested they play a role in brain development. Thus, changes to the expression and/or activation of Toll-like receptors may alter developmental outcomes. [76-78]

Activation of Toll-like receptors increases production of circulating inflammatory mediators such as cytokines. Cytokines are cell-signaling proteins produced by immune cells that function to eradicate infection and remove cellular debris, thereby promoting a healthy environment for development. However, cytokines are also involved in normal physiological processes such as blastocyst implantation and brain development. Cytokines can act on cells through autocrine, paracrine, and endocrine means. Proinflammatory cytokines include interleukin (IL)-1β, IL-6, IFN-γ, and tumor necrosis factor (TNF)-α, while anti-inflammatory cytokines include IL-1 receptor antagonist, IL-10, and transforming growth factor (TGF)-β. It is currently not clear whether maternal cytokines can gain access to the fetus. Zaretsky et al. investigated the transfer of cytokines by analyzing the clearance index (the ratio of transplacental transfer of cytokines to antipyrine) of perfused placentas. [79] Ten placentas were collected following caesarean deliveries and were allocated to one of three groups for analyses; maternal-fetal transfer (n=4), fetal-maternal transfer (n=2), and control. [79] The authors assessed the transfer capacity of IL6, IL-1α, and TNF-α and found that IL-6 was transferable between maternal and fetal circulations while IL-1α and TNF-α were not. [79] Girard and Sebire injected pregnant rats

with LPS on gestational day 18 every 12 hours up until gestational day 20, where rats were delivered with intravenous radiolabeled IL-1β. [80] Rats were euthanized after 30 minutes and placentas were harvested for detection of IL-1β. [80] The authors found minimal passage of IL-1β across the placenta. [80] The data suggest that changes in fetal IL-6 levels may be of maternal origin whereas IL-1β is unlikely to be derived from the maternal system. Once the fetus is exposed to inflammatory mediators it will mount a FIR, defined as an increase in plasma IL-6. [81] Cytokines can gain access to the fetal brain through the blood brain barrier, [82] circumventricular regions, [82] and/or stimulation of receptors found on the endothelium. [82] Cytokines will trigger microglial activation, the resident immune cell of the brain, which have both cytokine receptors and Toll-like receptor-4. [83-85] Activated microglia are able to release IL-1 and TNF-α, which in turn, stimulate astrocytes. [86-88] Astrocytes also produce TNF-α and IFN-γ. [89,90] TNF-α causes cell death of oligodendrocytes while IL-1β results in oligodendroglial cell loss and disruption of maturation. [91,92] [92,93] IFN-γ induces cell death through activation of enzyme inducible nitric oxide synthase which produces reactive nitrogen species and TNF-α, and also favours the transformation of microglia to an active pro-inflammatory state. [38,94] [95] Pang et al. found that LPS administration to microglia and oligodendrocyte progenitor co-cultures transformed the microglia to their M1 phenotypes and resulted in oligodendroglial injury via oxidative stress (24h), followed by cytokine toxicity (48h). [96] Lee et al. found that LPS was required to stimulate microglial production of IL-1β, TNF-α, and IL-6 cytokine mRNA (measured by northern blots) and protein levels (measured by enzyme linked immunosorbent assay) whereas IL-1β was required to activate astrocytes to produce TNF-α and IL-6. [97] These studies emphasize cell specificity and temporal changes in cytokine induction, which create difficulty identifying appropriate interventions to target the inflammatory cascade. Unfortunately, the complete

elimination of cytokines is unfavourable, as they are involved in normal development by promoting proliferation and differentiation of precursor cells. [98-100] Meyer et al. found that mice genetically manipulated to overexpress the anti-inflammatory cytokine IL-10 had minimized behavioural deficits induced by mimicking a prenatal viral infection with polyriboinosinic-polyribocytidilic acid (2 mg/kg intravenous injection on gestational day 9). [101] However, in mice that were not challenged with viral infection, IL-10 alone also induced behavioural anomalies. [101] Thus, imbalances in pro- and anti-inflammatory cytokines can be detrimental to the developing brain, and identifying therapeutic interventions that maintain cytokine balance is critical. [101]

1.3.4. Animal Models of Maternal Infection and Inflammation

Many animal models have been developed for the study of *in utero* inflammation. Several investigators have developed models of infection using microorganisms commonly observed in the clinic. [102,103] Debillon et al. inoculated pregnant rabbits with *Escherichia coli* (*E. coli*) and found increased inducible nitric oxide synthase immunostaining in the placentas and fetal brains, macrophages in the placentas, cystic periventricular white matter injury, and cell death in the hippocampus of the fetal brains (n=6 fetuses from 2-3 litters). [104] When *E. coli* was inoculated into pregnant rats, astrogliosis was observed in the hippocampus, along with a decrease in the myelin enzyme 2',3'-cyclic nucleotide phosphodiesterase, and the presence of neurofilament in the white matter, following a transient increase in chemokines. [105] Inoculation with *Ureaplasma parvum* increased expression of placental cytokines, microgliosis, and reduced the number of mature neurons. [106] The administration of the microorganism without antibiotics resulted in higher rates of prematurity and stillbirths, a confounding factor in these studies. [107] Originally, a

correlation between bacteremia and white matter injury in infant (ages 9-30 days) autopsy brain records was identified; however, despite positive blood cultures there was no evidence of bacteria within the brain, suggesting a role for cytokines. [55,108,109] To identify the role of cytokines, LPS was systemically administered to neonatal kittens (*n*=8 litters). [56] Widespread astrogliosis and cystic necrosis were detected following immunohistochemical and hematoxylin and eosin staining, reflecting cystic periventricular leukomalacia. [56] This has led to the use of LPS to elicit an inflammatory reaction, rather than bacteria. The doses of LPS used range from the 0.05 mg/kg, which enhanced proinflammatory messenger RNA (mRNA) levels, to 12 mg/kg, which increased proinflammatory cytokine levels. [82,107,110] The diversity of protocols developed to induce inflammation results in variations in the severity of the resultant brain damage, but consistently involve the periventricular white matter. Data from experimental studies has established that maternal inflammation can cause significant brain damage in newborns. It is important to consider that chorioamnionitis originates in the maternal system. This highlights the importance of understanding the effects of inflammation on the maternal system during pregnancy as well as its contribution to fetal brain injury.

1.4. PHYSIOLOGY OF PREGNANCY

1.4.1. Immunology of Pregnancy

Pregnancy is an active immunological state and chorioamnionitis involves alterations to the immune system. The immune system in a non-pregnant individual is highly effective in identifying self from non-self. The innate immune system is the first line of defense,

characterized by macrophages and natural killer cells, and responds within hours. Adaptive immunity is characterized by the major histocompatibility complex, as well as B (humoral) and T (cell mediated) lymphocytes, and responds within days-weeks. To effectively expel harmful particles, T lymphocytes must detect them via antigen receptors, which result in activation of T lymphocytes and production of cytokines (discussed above). T lymphocytes are categorized into two groups based on the cell surface antigens, CD4 or CD8. CD4 (otherwise known as helper T cells) can be further subcategorized into Th1 and Th2, categorized as pro-inflammatory and anti-inflammatory, respectively. Following activation of CD4 cells, B cells are then activated to "memorize" the antigen for effective and rapid removal upon subsequent exposure.

Pregnancy poses a conflicting circumstance, as the foreign fetus must be able to survive and grow within the mother. The immune system must therefore accept the developing fetus, an immunological state referred to as tolerance, while still being active to protect the mother against infection. [111] Pregnancy represents a dynamic and active immunological state, involving complex interactions between T helper 1 cells, T helper 2 cells, T helper 17 cells, and regulatory T cells, to establish and maintain pregnancy. [112] [113] During pregnancy, there is a predilection towards T helper 2 cells over T helper 1 cells type. In addition, a subset of T cells that produces IL-17, referred to as T helper 17 cells, promote inflammation whereas T regulatory cells counteracts T helper 17 cells by suppressing inflammation. [111,114] Furthermore, induction of T helper 17 cells and regulatory T cells is mediated by the pro-inflammatory cytokine IL-6, and the anti-inflammatory cytokine TGF-β, respectively. [111] An appropriate cytokine balance is required to facilitate fetal growth and prevent rejection. The inflammatory state during pregnancy is highly

dynamic and alterations to it, as may occur in the setting of chorioamnionitis, can lead to adverse pregnancy outcomes.

1.4.2. Uterine Vascular Adaptations

In early pregnancy, there is an increase in blood volume to support the uterus, placenta, and fetus, along with structural and functional changes to the uterine vasculature. [115] The uterine vasculature grows to accommodate larger volumes of blood flow by expanding lumen diameter, lengthening blood vessels, and reducing resistance. [115] Poiseiulle's law comes into play when considering the relationship between diameter, length, and resistance. [115] Poiseiulle's law states that the increase in diameter by a certain length (referred to as *x*), will cause resistance to be the inverse of *x* raised to the power of four; where as a change in length is proportional to the change in resistance. [115] The uterine vasculature expands via several mechanisms, including exposure to early gestational hormones, smooth muscle cell hypertrophy and hyperplasia, and growth factors. [115] Enlargement of the endothelial lumen augments blood flow, which causes shear stress and pressure against the endothelial cell walls, thereby stimulating release of vasoactive substances that influence vessel tone. [115,116] Together with vascular structural and functional changes, these adaptations are important for the growth and survival of the fetus.

1.4.3. Regulatory Mechanisms of Vascular Tone

Endothelial cells are capable of producing vasoactive substances to modulate vascular tone. Vasodilators include NO, prostacyclin, and endothelium derived hyperpolarizing factor.

Vasoconstrictors include endothelin-1, thromboxane A_2, and angiotensin II. [117] During pregnancy, the uterine vasculature has increased responsiveness to vasodilators (i.e., NO) and decreased receptivity to vasoconstrictors (i.e., angiotensin II). Concentrations of NO metabolites (i.e., nitrates and nitrites) in the urine are also elevated during pregnancy, suggesting a predominant influence of NO on the vasculature. NO is produced from the conversion of L-arginine to NO and *L*-citrulline, by the enzyme endothelial nitric oxide synthase. NO diffuses into smooth muscle cells and stimulates conversion of guanosine triphosphate to cyclic guanosine monophosphate, via guanylyl cyclase. Cyclic guanosine monophosphate decreases intracellular Ca^{2+}, leading to relaxation of vascular smooth muscle cells and vasodilation. There are three isoforms of nitric oxide synthase: endothelial nitric oxide synthase and neuronal nitric oxide synthase which are Ca^{2+} dependent, and inducible nitric oxide synthase, which is Ca^{2+} independent. Prostacyclin is also a vasodilator and is produced from the conversion of arachidonic acid by cyclooxygenase (-1 or -2) to prostaglandin H2. Prostaglandin H2 is converted to prostacyclin via prostacyclin synthase. Prostacyclin then moves into smooth muscle cell where it activates cyclic adenosine monophosphate, which also leads to a reduction in Ca^{2+}, and vasodilation. Endothelial cells can also produce endothelial derived hyperpolarizing factor via phospholipase A_2 metabolism into epoxy-eicosatrienoic acids, causing K^+ channel hyperpolarization, and smooth muscle relaxation.

Increases in nitric oxide synthase (i.e., endothelial nitric oxide synthase levels) and cyclic guanosine monophosphate levels in uterine arteries have been identified during pregnancy. Magness et al. investigated the presence of nitric oxide synthase and cyclic guanosine monophosphate in uterine, renal, and omental arteries of nonpregnant (n=11) and pregnant

(*n*=24) ewes. [118] The authors found that endothelial nitric oxide synthase was evident in the endothelial layer of all three arteries, and was greatest in the uterine artery from pregnant ewes. [118] Furthermore, plasma and urinary cyclic guanosine monophosphate levels positively correlated with uterine artery endothelial nitric oxide synthase levels. [118] A similar study by Nelson et al. analyzed uterine arteries from pregnant (*n*=15) and non-pregnant (*n*=30) patients undergoing hysterectomy. [119] The authors found that exposure to Ca^{2+} increased arginine metabolites in uterine arteries from pregnant women, suggestive of Ca^{2+} dependent NOS enzyme activity, whereas incubation with L-N^G-nitroarginine methyl ester (L-NAME), a pan nitric oxide synthase inhibitor, reduced the production of these metabolites. [119] Endothelial nitric oxide synthase localized to the endothelium was shown to be upregulated, and an increase in NO-dependent cyclic guanosine monophosphate levels was detected in arteries from pregnant animals, compared to arteries from non-pregnant animals. [119] Overall, these studies support the involvement of the NO-cyclic guanosine monophosphate pathway in mediating vascular tone during pregnancy.

Several agonists induce vasoconstriction, including endothelin-1 and angiotensin II. Endothelin-I is a peptide produced by endothelial cells. [120] Endothelin-I arises from pre-pro-endothelin-I, which is converted to big endothelin-1, and subsequently undergoes enzymatic cleavage by endothelin converting enzymes. [120] Endothelin-I binds to endothelin receptor type A and endothelin receptor type B. [120] These receptors are found on smooth muscle cells, which mediate vasoconstriction. [120] However, endothelin receptor type B has also been identified in endothelial cells and activation of this receptor favours relaxation. [120] Endothelin-1 has also been shown to activate the transcription factor NF-κB, which can in turn activate transcription of

endothelin-1. [121,122] Another vasopressor agent leading to vasoconstriction is angiotensin II. Angiotensinogen is converted to angiotensin I, and then to angiotensin II, by angiotensin-converting enzyme. Angiotensin II binds to either angiotensin II receptor type 1 or angiotensin II receptor type 2, which mediate vasoconstriction and vasodilation, respectively. Following binding to angiotensin II receptor type I, phospholipase C is activated, an enzyme that produces inositol-1,4,5-trisphosphate and diacylglycerol. [123] Inositol-1,4,5-trisphosphate increases the concentration of calcium in the cell, which ultimately leads to contraction. [123] Activation of angiotensin receptor type II can also produce bradykinin, which can stimulate endothelial nitric oxide synthase to produce NO, mediating relaxation. [123] Similar to endothelin I, angiotensin II is also able to activate the transcription factor NF-κB and NF-κB can initiate transcription of angiotensin II. [124,125] The balance between vasorelaxation and vasoconstriction is a dynamic process that requires an appropriate balance between these endothelium-derived factors. In addition, these vasoconstrictors have been shown to play a role in the inflammatory process, which can generate oxidative stress through downstream signaling. This can ultimately impair vascular reactivity if not properly restored to physiological conditions.

During pregnancy, the vasculature is less responsive to vasoconstrictors. Gant et al. evaluated the response to the vasoconstrictor angiotensin II in 192 pregnant and 10 non-pregnant females. [126] The authors found that decreased responsiveness to angiotensin II occurred as early as 18 weeks post-conception, followed by a slight decline after 30 weeks. [126] Interestingly, in pregnant women who developed hypertension, the resistance to angiotensin II was maximal by 18 weeks and declined thereafter. [126] This study highlights that the modulation of vascular tone becomes less responsive to vasoconstrictors in healthy pregnancies. Hermsteiner et al. tested

uterine and mesenteric artery contractile responses to phenylephrine, angiotensin II, and endothelin I, in non-pregnant and pregnant Sprague Dawley rats (early pregnancy, gestational day 7-9 and late pregnancy, gestation day 19-21). [127] In uterine arteries isolated from early (*n*=5) and late pregnancies (*n*=4), phenylephrine response was suppressed compared to arteries from non-pregnant rats (*n*=4), whereas in the mesenteric arteries, only late pregnant vessels (*n*=9) were less sensitive to the effects of phenylephrine compared to early pregnant (*n*=9) and non-pregnant (*n*=22). [127] When the vessels were incubated with angiotensin II, the uterine arteries isolated from early pregnant rats (*n*=5) had a reduced response to the constrictor, whereas the late pregnant (*n*=8) and non-pregnant (*n*=13) vessels did not differ in their responses. [127] In the mesenteric arteries, non-pregnant (*n*=30), early pregnant (*n*=5), and late pregnant responses (*n*=7) did not differ. Vasoconstrictor responses to endothelin I were reduced in late pregnant uterine arteries (*n*=5), however, this was not observed with early pregnant (*n*=5) and non-pregnant (*n*=5) arteries. [127] No differences in response were observed in the mesenteric arteries between non-pregnant (*n*=6) and pregnant states (*n*=5/group). [127] This study conveys the complex interaction between vasoconstrictors, non-pregnant versus pregnant physiological states, and gestational age, in modulating vascular tone.

1.4.4. Inflammation and Oxidative Stress Alters Vascular Tone

Disturbances to pregnancy-induced vascular modifications can cause adverse effects such as intrauterine growth restriction (Figure 1.1). Such alterations can be induced following maternal infection and inflammation, including chorioamnionitis. Munshi et al. administered LPS to human umbilical vein endothelial cells and tested for DNA fragments, chromatin

cleavage, and caspase activity, all of which are indicative of apoptotic cell death. [128] LPS stimulated caspase-1 and -3, p53, increased mitochondrial Bax levels, and reduced Bcl-2 and focal adhesion kinase proteins. [128] Overall, the authors demonstrated a time-dependent effect of LPS-induced apoptotic cell death, where p53 and Bax were increased between 3-6 hours, followed by caspase-3 at 6-12 hours. [128] Orshal and Khalil infused pregnant Sprague-Dawley rats (*n*=6-8/group) with IL-6 (200 ng/kg/day) over a period of 5 days beginning on E15. [129] IL-6 infused pregnant rats had increased blood pressure, enhanced contraction of aortic vascular strips in response to phenylephrine, reduced vasorelaxation in response to acetylcholine, and reduced endothelial nitric oxide synthase protein levels compared to non-infused pregnant, IL-6 infused non-pregnant, and non-infused non-pregnant rats. [129] The lack of alterations to vascular responsiveness in endothelium-denuded aortic rings suggested that the effects of pro-inflammatory cytokines are mediated by endothelial dependent mechanisms (i.e., NO production). [129,130] Oxidative stress can occur secondary to inflammation and together they can augment vascular damage and induce the continuation of a vicious cycle. Pro-oxidants such as superoxide anion can bind to NO and reduce bioavailability. Ulker et al. evaluated the role of oxidative stress and the effects of vitamins C and E on endothelial function of thoracic aortas isolated from spontaneously hypertensive male rats (*n*=4-6). [131] The authors found that treatment with vitamin C and E was effective at decreasing superoxide production via suppression of nicotinic adenine dinucleotide phosphate oxidase. [131] These effects were paralleled by an increase in endothelial nitric oxide synthase activity (which can be suppressed by inflammatory cytokines) and NO, thereby restoring vasodilation. [131] This study highlights the involvement of oxidative stress in endothelial dysfunction and the normalization induced by the scavenging of free radicals. Okatani et al. collected human umbilical arteries (*n*=10) between 37-39 weeks post

conception and evaluated the effects of homocysteine-mediated oxidative stress and the protective effects of the hormone melatonin on vascular function. [132] Oxidation of homocysteine produces the pro-oxidants superoxide anion and hydrogen peroxide, which can induce endothelial damage. [132] Melatonin as a free radical scavenger that can also promote production of antioxidant enzymes may be a suitable agent to counteract the pro-oxidants produced by homocysteine. [132] The authors exposed the arteries to increasing doses of serotonin, and found enhanced constriction following preincubation with homocysteine, whereas pretreatment with melatonin (1 and 10 μM) reduced the enhanced pressor response. [132] In summary, under pathological conditions such as chorioamnionitis, circulating cytokines may augment vasoconstriction and impair vasorelaxation. Furthermore, inflammatory conditions can induce oxidative stress and cell death, which may contribute in part to the aberrant vascular activity.

1.4.5. Development of the Placenta

The placenta is the interface between the mother and fetus. It functions as a physical and immunological barrier and also transports oxygen, nutrients, and other substrates to facilitate fetal growth and homeostasis, and removes waste. Placentation occurs following implantation of the blastocyst into the endometrium. Trophoblast cells surrounding the blastocyst differentiate into an inner cell layer, the cytotrophoblast, and an outer cell layer, the syncytiotrophoblast. [133] Cytotrophoblast cells undergo proliferation, forming cells that will lose their membrane and merge to form the syncytiotrophoblast layer, which invades maternal tissue, and cytotrophoblast cells that will invade into the syncytiotrophoblast layer to form chorionic villi. [133] Fetal trophoblast cells invade the spiral arteries, a process called endovascular invasion, transforming

the spiral vessels to lower resistance and widening of arteries proximal to the lacunaes. This increases maternal blood flow to the lacunaes of the placenta, forming the intervillous space. The extraembryonic mesoderm penetrates the chorionic villi, followed by evagination of fetal blood vessels within the mesoderm. [133] Maternal blood surrounds the chorionic villi, and maternal and fetal blood exchange gasses and nutrients through diffusion, but maternal and fetal circulations do not come into direct contact. The placenta represents an important organ that facilitates communication between mother and fetus, protects the fetus, as well as provides nutrients and growth factors to facilitate fetal growth and development.

1.4.6. Utero-Placental Inflammation

The placenta is also a highly active immunological site, and is exposed to both maternal and fetal immune mediators. The maternal portion of the placenta is referred to as the decidua. It consists of uterine natural killer cells, macrophages, regulatory T cells, and dendritic cells. [111,112,134] Uterine natural killer cells regulate spiral artery invasion and have a reduced cytotoxic function, allowing the fetus to escape rejection. [135] Macrophages are responsible for repair and renewal, thus facilitating and maintaining pregnancy, especially during implantation when several cell structures are damaged. [112, 136] The fetal component of the placenta, the trophoblast cells, also play a role in immunoregulation, expressing Toll-like receptors, Fas ligand, and the major histocompatibility complex class I and G, which detect microorganisms, induce apoptosis in maternal immune cells, and escape cell death, respectively. [112,137-141] An increase in pro-inflammatory cytokines IL-1β, TNF-α and IL-6 has been detected in the placenta, [142-147] and has been linked to brain damage and fetal death. [148-151] Systemic maternal inflammation, which can

result from chorioamnionitis, induces placental damage through the elevation of inflammatory mediators, resulting in placental underperfusion and fetal brain injury. [148] Accordingly, treatment with IL-1 receptor antagonist, an inhibitor of the pro-inflammatory cytokine IL-1β, protected the placenta from cell death following underperfusion. Prevention of placental inflammation may be able afford downstream protection of the fetal brain against neurodevelopmental disorders. [148]

1.5. BRAIN DEVELOPMENT

1.5.1. Brain Development

The development of the neocortex can be differentiated into three distinct periods: proliferation, migration, and differentiation. [152-157] Following conception, the embryo undergoes gastrulation forming three germ layers: ectoderm, mesoderm, and endoderm. [152-157] The ectoderm layer subsequently gives rise to the neural tube. The neural tube is composed of progenitor cells; those lying in the most rostral segment will form the brain, the caudal end will become the spinal cord, and the hollow portion will be the ventricular system. [152-157] The progenitor cells are localized immediately adjacent to the ventricles, and this region is referred to as the ventricular zone. [152-157] The rostral end of the tube begins to swell, forming gyri and sulci, which ultimately regionalize the brain. [152-157]

Signaling and immune proteins such as the leukemia inhibitory factor, ciliary neurotrophic factor, and TGF-β regulate neurogenesis. [158] Neurogenesis begins with proliferation in the ventricular zone. [158] Neural progenitor cells undergo several rounds of symmetrical mitotic

divisions to increase the population pool, followed by asymmetrical mitotic division or horizontal cleavage. [158] Daughter cells that lie above the cleavage line will migrate into the cortex and mature into neurons. [158] Daughter cells that lie below the cleavage line will become progenitor cells, continuing to undergo several divisions of mitosis to maintain the pool of progenitors. [158] The first wave of newly formed neurons migrates above the ventricular zone to form the preplate. [158] This preplate is subsequently split into two layers: the marginal zone and subplate, both of which are transient layers. [158] A third layer, the cortical plate, forms between the marginal and subplate zone, which contains the six layers of the neocortex. [158]

The growth of the cortex requires appropriate guidance to each destined region of the brain. [152-157] Neural progenitor cells can subdivide to produce radial glial cells. [152-157] Radial glia cells extend their cytoplasm to the pial surface of the brain, while their cell body remains in the ventricular zone. [152-157] This provides a 'scaffold' for newly formed neurons to migrate along to their destined location. [152-157] Cues are also provided to direct the migration of newly born neurons to the appropriate cortex layer. [152-157] Cajal-Retzius cells reside in the marginal zone and produce guidance cues for newly formed neurons. [152-157] These cells produce a signaling molecule, reelin, that directs the migratory destination of neurons. [152-157] The cortex develops in an 'inside-out fashion' where the oldest cells contribute to the deep layers of the cortex, such as layers V and VI, while the younger cells inhabit the superficial layers of the cortex, such as layers I and II. [152-157] Furthermore, a second proliferative site, the ventral telencephalon, produces inhibitor *gamma*-aminobutyric acid interneurons. [152-157] Interneurons use tangential migration that involves traversing the cortex, rather than radial migration, and these cells use semaphorins as the signaling molecules, as opposed to reelin. [152-157]

Following migration, differentiation occurs. [152-157] Early progenitor cells are capable of producing any neuronal subtype, but as development proceeds, these progenitor cells become 'fate restricted'. [152-157] Differentiation involves extension of several cytoplasmic processes from the neuronal cell body into dendrites, branch-like structures that receive input from neighbouring neurons. [152-157] On the opposite side of the cell body, the neuron develops a single axon, a long projection that surveys the area for guidance cues to establish connections with its target. [152-157] The next process is termed synaptogenesis. [152-157] During synaptogenesis, once the target is established, the axon will form a presynaptic bouton, and the targeted dendrite will be signalled to develop the postsynaptic terminal. [152-157] Communication is established by transmission of specific chemical messengers, differing in each subtype of neuron, and an action potential is propagated down the axon. [152-157] These synapses are constantly active throughout life and are dependent on experience-based plasticity. [152-157] Once these connections are made, cortical wiring is established. [152-157] Finally, programmed cell death via apoptosis occurs to remove excess and non-essential neurons, and those forming inappropriate circuitries. [152-157]

The switch to gliogenesis involves activation of the Janus kinase/signal transducer and activation of transcription signaling pathway, expression of proteins such as bone morphogenic protein, and methylation of gliogenic promoter regions. [153,159] Glial cells include astrocytes and oligodendrocytes. [153,159] Astrocytes are a supporting cell with several roles including trophic factor and blood vessel wall support, synaptogenesis, and ion homeostasis. [153,159] Oligodendroglial cells are involved in myelination. Myelination aids in increasing the efficiency of action potentials and provides trophic factors to maintain axons and promote growth. [153,159] The last type of cell found in the brain is microglia, which are the only non-neural cells found in

the brain. [153,159] The microglial cells are the immune cells of the brain. [153,159]Microglia, under the instructions of growth factors, transcription factors etc., undergo proliferation and differentiation. [153,159] Microglia can also modulate neuronal survival and death by releasing neurotrophic factors, cytokines, and pro-oxidants. [153,159] These cells also act in concert to facilitate maturation of one another and ensure proper synaptogenesis. [153,159]

1.5.2. Cytokine Regulation of Brain Development

Cytokines are found in small concentrations in the brain, facilitating proper growth, migration, and differentiation, and are spatiotemporally regulated. [160] In addition to fetal systemic sources of cytokines, neurons and glial cells can also produce cytokines and chemokines. [97,161] Cytokines involved in development are referred to as neuropoietic (Figure 1.2). [158,162] This family of cytokines include IL-6, ciliary neurotrophic factor, leukemia inhibitory factor, IL-11, and cardiotrophin-1, among many others. [163,164] These cytokines share a common transmembrane receptor subunit, glycoprotein 130, and are sometimes referred to as the gp130 family of cytokines. [163,164] Depending on the type of ligand, activation by a cytokine induces the glycoprotein 130 subunit to form a homo- or heterodimer that facilitates activation of the Janus kinase, followed by phosphorylation and activation of signal transducer and activation of transcription (-3 or -1). [163,164] Phosphorylation of signal transducer and activation of transcription-3 promotes neuronal cell survival, but is also capable of suppressing neurogenesis in favour of gliogenesis, whereas signal transducer and activation of transcription-1 is involved in maintenance of astrocytes. [165-167] Activation of the leukemia inhibitory factor receptor by leukemia inhibitor factor and ciliary neurotrophic factor have been shown to maintain neural

progenitor proliferation and cortical organization. [168] Cytokines such as members of the TGF-β family, the bone morphogenic proteins, are involved with neuronal induction and differentiation, and the onset of gliogenesis. [158,169-171] Once astrocytic proliferation is complete, the progenitors are signalled to produce oligodendrocytes via interaction of the IL-6 and TGF-β superfamily. [158,172] Oligodendroglial migration is controlled in part by astrocytic expression of the chemokine (C-X-C motif) ligand 1. [158]In summary, while cytokines can cause brain injury, they also perform an important role in facilitating brain maturation.

1.5.3. Neurotrophic Factors

The placenta provides neurotrophic factors for brain development. [173,174] The placenta initially produces high concentrations of these growth factors, but production declines as gestation progresses, as the fetus becomes capable of producing endogenous growth factors. Neurotrophic factors such as brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin 3 (NT-3), are polypeptides heavily involved in the proliferation, differentiation, and maintenance of cells. [175, 176] Neurotrophic factors bind to tropomyosin (or tyrosine) receptor kinase receptors: BDNF binds to tyrosine kinase receptor B, NGF binds to tyrosine kinase receptor A, and NT-3 binds to tyrosine kinase receptor C. Another receptor that can bind to any of the neurotrophic factors is the p75 neurotrophin receptor signaling cell death via apoptosis. Altered neurotrophin concentrations in the placenta can also influence growth restriction in the offspring. [177,178] Regulation of neuronal development involves activation of tyrosine kinase receptor A, which results in proliferation and differentiation via neuronal growth cone and migration, as well as regulation of enzymes involved in neurotransmitter formation.

Tyrosine kinase receptor B signaling is associated with cell survival, neuronal growth, and plasticity. Tyrosine kinase receptor C activation induces neuronal and oligodendroglial cell survival and proliferation. Experiments involving knockout of neurotrophic factors demonstrate loss of somatosensory and motor neurons. [179] Wirth et al. extracted pyramidal cells and assessed dendritic morphology following overexpression of BDNF and NT4/5. [180] Overexpression of either neurotrophic factor enhanced dendritogenesis and length; however; only removal of NT4/5 diminished the complexity of pyramidal neurons. [180] Neurotrophic factors can function as attractive or repulsive cues, guiding axonal growth cones to their appropriate target. [181-183] They also play a role in learning and memory by influencing long-term potentiation and synaptogenesis, [184-186] and regulating neurotransmitter release. [187-189] Interestingly, concentrations of placental BDNF, NGF, and NT-3 were increased in pre-eclampsia and chorioamnionitis, compared to control. [190-192] Thus, these alterations can lead to downstream changes to brain development. Overall, neurotrophic factors influence brain development from very early in life, and have a sustained influence into adulthood.

1.5.4. Rodent Brain Development

Our understanding of brain development relies on experimental models including rodent models, [193] the most commonly used of which is the rat. [194] Rats have been selected as the animal model of choice for several reasons, including their quick gestation, multiple offspring, cost effectiveness, and the now extensive literature available on rat brain development. Although the brains of rats are lissencephalic (lack of gyri and sulci), a study by Finlay and Darlington identified that neurogenesis is a conserved developmental process across species. [195] The

gestational period of humans is 40 weeks; in rats it is approximately 21-23 days. Newborn rat pups are considered to be altricial, meaning that they are underdeveloped when born. Humans newborns are somewhere between altricial and precocial (mature), although their neurological development favours the altricial side. The study of brain development in rats began with a study by Dobbing and Sands, where they characterized the brain volumes of humans and rodents at different gestational ages. [196] The authors concluded that at postnatal day (PD) 7, the rat has a brain equivalent to a human term infant, and that the brains of newborn rats are equivalent to those of premature infants. [196] The rat brain at 3 weeks of age is believed to correspond to that of a 2-year-old human. [193,196] This implies that the first trimester of human brain development is parallel to the first half of gestation in rats, the second trimester resembling the remaining gestational period, and the third trimester to correspond to the first week of life in rats. [196] However, several studies have identified that there are specific periods of neural development that may differ across brain substructures. [194]

Neurogenesis occurs at the end of the first week of pregnancy in rats, and can continue until PD15. [193,197] Following neurogenesis, neuronal maturation occurs. In humans, synaptogenesis peaks at 2 years of age, and is completed in adolescence. In rats, synaptogenesis peaks at 2 weeks, and is completed by the fourth week of postnatal life. [193,198,199] Romijn et al. found that human term newborn synapse formation is equivalent to that of a PD15 rat pup, glutamate decarboxylase expression to that of a PD16 rat pup, choline acetyltransferase to that of a PD20 rat pup, and electrical activity to that of a PD12-13 rat pup. [200] Following neurogenesis, neural stem cells begin gliogenesis, resulting in the production of astrocytes and oligodendrocytes. At term, astrocytes will begin to express the glial fibrillary acidic protein

whereas in rodents, minimal expression is observed on gestational day 16. [193,201] In humans, pre- and immature oligodendrocytes are dominant between 18-28 weeks and 28-40 weeks gestation, respectively. [193,202] In rats, pre- and immature oligodendrocytes predominate around PD2 and PD5-7, respectively. [193,202,203] Microglial cells can be found to localize in the brain parenchyma at about 16 weeks and gestational day 12 in the human and rat, respectively. [204] Thus, the animal model used should be carefully considered when timing insults.

1.6. THERAPEUTIC INTERVENTIONS

Substantial advancements have been made in maternal and neonatal care, such that the incidence of surviving preterm infants is increasing. [205] However, preventive interventions for infants at risk of developing CP are lacking. Several therapeutic interventions have been investigated to determine their efficacy in treating inflammation and preventing CP; but none have been successfully translated into clinical practice. For example, adult medications such as NMDA antagonists used to treat ischemic injury have been shown to be harmful to the developing fetal brain. [206] Identifying therapeutic interventions has been unsuccessful for several reasons. First, the developing brain is complex and difficult to treat. As mentioned, the brain is considered a 'moving target' because the developmental stage changes over time. The onset, duration, and severity of brain injury are not easily elucidated following imaging scans, which are used to detect pathophysiological damage. Moreover, there are moral and ethical concerns regarding drug delivery to a pregnant women and whether or not a drug will reach its target due to the numerous physiological barriers. These are just a few obstacles to identifying optimal therapeutic interventions.

Antibiotics are given to pregnant women with clinical symptoms of chorioamnionitis in an effort to eradicate the organism as well as prolong gestation. In a study by Gravett et al., the use of ampicillin (*n*=4), or ampicillin in combination with immunomodulators dexamethasone and indomethacin (*n*=5) in pregnant rhesus monkeys (*Macaca mulatta*) increased gestational length and suppressed inflammatory cytokines (IL-1β, TNF-α, prostaglandin E_2, and prostaglandin $F_{2\alpha}$) in comparison to those that did not receive antibiotics (*n*=6).[207] However, the authors did identify that antibiotic and anti-inflammatory treatment may be given too late in a clinical setting, as pregnant women may not know they have an infection.[207] This highlights the need to be able to identify 'at risk' patients and the important role of inflammation following infection. Interestingly, prophylactic antibiotic treatment was found to be associated with an increase in neonatal deaths.[208,209] King et al. and Flenady et al. found that prophylactic antibiotics were effective in treating infection; however, certain antibiotics were associated with adverse outcomes in the newborn, including mortality.[208,209] Antibiotic treatment may not be effective for all cases of chorioamnionitis.

N-acetylcysteine is an amino acid involved in the generation of glutathione. *N*-acetylcysteine has been shown to be safe and capable of traversing the placenta, while its ability to cross the blood brain barrier is controversial.[210-212] In a model of inflammation induced by LPS injections and in a double hit model of both LPS and hypoxia, treatment with *N*-acetylcysteine reduced oxidative stress, inflammation, and promoted expression of antioxidant enzymes.[213-215] Furthermore, oral *N*-acetylcysteine given to pregnant women resulted in prolonged gestation.[216] Unfortunately, despite these potential benefits, *N*-acetylcysteine has been shown to aggravate LPS-induced cardiovascular abnormalities in a sheep model of inflammation.

[217] Probyn et al. measured fetal lamb arterial pressure and heart rate following catherization and infusion with saline (*n*=6), LPS (*n*=6), LPS + *N*-acetylcysteine (*n*=6), and *N*-acetylcysteine alone (*n*=3). [217] Treatment with *N*-acetylcysteine was found to augment hypoxemia and hypotension in LPS-exposed fetal lambs, but was not harmful when given to saline-infused fetal lambs. [217] The authors hypothesized that administration of *N*-acetylcysteine may delay the development of tolerance to LPS; however, why *N*-acetylcysteine augmented the deleterious effects of LPS remains unknown. [217]The authors highlighted that *N*-acetylcysteine was given following an inflammatory response, whereas a protective response was observed in a study where *N*-acetylcysteine was given prior to the insult. [217,218] This suggests that *N*-acetylcysteine may potentiate the actions of LPS when given as an intervention but not when given prophylactically. [217]

The use of anti-inflammatory cytokines has also been investigated to determine their possible protective effects under conditions of *in utero* inflammation. Girard et al. injected pregnant rats with LPS and observed placental damage along with elevation of proinflammatory cytokines IL-6, TNF-α, and IL-1β. [143,148] When IL-1 receptor antagonist was given 12 hours after LPS injections, placental damage was ameliorated. [143,148] Furthermore, IL-1 receptor antagonist treatment prevented behavioural abnormalities, neural stem cell loss, white matter injury, and gliosis in offspring exposed to an inflammatory *in utero* environment. [143,148] Treatment with IL-1 receptor antagonist occurred after the inflammatory insult; this regimen has yet to be investigated as a prophylactic intervention. The anti-inflammatory cytokine IL-10 has also been investigated as a potential therapeutic agent. Meyer et al. reported beneficial effects in offspring following genetic manipulation resulting in continuous overexpression of IL-10 in a

model of maternal viral infection. [101] However, overproduction of IL-10 in the normal physiological environment as a prophylaxis *in utero* altered the behaviour of offspring. [101] These studies suggest that pro-inflammatory and anti-inflammatory cytokines are maintained in a homeostatic balance, and manipulating this balance may not be beneficial.

1.7. BROCCOLI SPROUTS (BrSp)

A possible therapeutic intervention is the consumption of a healthy diet. In general a healthy diet is important at every stage and aspect of life. A diet rich in fruits and vegetables has been shown to reduce the risk of coronary heart disease, cancer, and stroke. [219-223] More specifically, cruciferous vegetables, which belong to the family *Brassicaceae*, contain phytochemicals that may be an effective therapeutic intervention for inflammatory conditions and have been associated with reduced risk of cancer and cardiovascular diseases. [224] Cruciferous vegetables are named as such due to their cross like shape and contain several phytochemicals that are referred to as natural health products. [225] Natural health products (also referred to as Natural and Non-prescription Health Products) are regulated under the Natural Health Products Directorate and is defined by Health Canada as "*Vitamins and minerals, herbal remedies, homeopathic medicines, traditional medicines such as traditional chinese medicines, probiotics, and other products like amino acids and essential fatty acids*". [225] The benefits observed are hypothesized to be due to the high concentrations of the sulphur-containing compounds called glucosinolates. [224,226] Glucosinolates generate the bitter flavour and distinct odor that occurs following consumption of these vegetables. Moreover, cruciferous vegetables also contain carotenoids, vitamins A, B, C, E, and K, folate, minerals (iron, magnesium, and

manganese), protein, omega-3 fatty acids, and fiber, all of which are beneficial to health. Upon mechanical breakdown either by chewing or food preparation, the glucosinolates are converted to isothiocyanates, indoles, and nitriles.[226] Thus, consumption of the whole vegetable may confer more benefits from the synergistic effects of the phytochemicals compared to isolated individual components.[227] Broccoli sprouts (BrSp) have been shown to contain the highest concentrations of glucoraphanin, a glucosinolate, compared to all other cruciferous vegetables, and their protective properties exceed those of their mature counterpart, broccoli.[226,228] These potent protective properties are afforded by the isothiocyanates, which contain the –N=C=S functional moiety, promoting endogenous antioxidant and anti-inflammatory activity.[226,229]

BrSp consumption has been shown to be advantageous in many experimental studies. Wu et al. fed BrSp (200 mg/day) to spontaneously hypertensive rats (*n*=8-10/group) and observed a reduction in oxidized glutathione, increased glutathione peroxidase and reductase, and reduced NF-κB protein levels in several tissues including aorta, carotid artery, heart, and kidneys.[230] Noyan-Ashraf et al. found that dietary supplementation with BrSp in spontaneously hypertensive rats (*n*=6-9/group) reduced blood pressure, increased glutathione levels, reduced oxidized glutathione levels, increased glutathione peroxidase and reductase activity, decreased inducible nitric oxide synthase, nitrosylated proteins, and NF-κB p65/IκBα protein levels in the offspring, suggesting that the outcomes may also confer benefits to the developing fetus.[231] These studies also suggest that consumption of BrSp suppresses inflammation and oxidative stress. Importantly, inflammation and oxidative stress are common denominators in many diseases, therefore the protective properties of BrSp may be extended to several pathologies.

1.7.1. Sulforaphane (SFN)

The phytochemical glucoraphanin is converted to SFN, both of which are natural health products, by the enzyme myrosinase. This enzyme only comes into contact with glucoraphanin following breakdown of the plant since it is separated from it by the cell wall. SFN is an isothiocyanate and potent inducer of endogenous phase II enzymes such as glutathione S-transferase and N-acetyltransferase.[229] Under homeostatic conditions, xenobiotics are metabolized by phase I enzymes (e.g., cytochrome P450) into electrophilic substances. These electrophilic substances may be harmful and thus are inactivated by phase II enzymes to facilitate removal from the body via excretion. Some of the phase II enzymes involved in this process are also involved in the generation of the major antioxidant found in the body, glutathione. However, in the developing brain, the production of phase II enzymes is limited due to its immature antioxidant system.[45] Consumption of cruciferous vegetables, which contain high concentrations of isothiocyanates, by pregnant women could facilitate production of endogenous phase II enzymes in the fetal brain.

The production of phase II antioxidant enzymes is initiated by the SFN activated transcription factor nuclear factor (erythroid-derived 2)-like (Nrf2) (Figure 1.3). In normal physiological conditions, Nrf2 is sequestered and targeted for degradation in the cytoplasm by the kelch-like ECH associated protein 1.[232] The kelch-like ECH associated protein 1 is associated with cullin 3, an enzyme that ubiquinates Nrf2. However, following activation by oxidative or electrophilic stress, or exposure to SFN, the bond between the two compounds is disrupted at cysteine or sulfhydryls residues found on kelch-like ECH associated protein 1.[233,234]

The kelch-like ECH associated protein 1 releases Nrf2, which is then free to translocate into the nucleus. SFN then heterodimerizes with the small maf transcription factors, and the complex binds to its cognate DNA promoter region, the antioxidant response element. Binding to the promoter region initiates transcription of phase II enzymes. Through this pathway, SFN enhances production of endogenous phase II and antioxidant enzymes level, resulting in cell protection. [235] Zhao et al. found that brain damage caused by transient blockage of both the left common carotid and middle cerebral arteries was reduced in rats receiving systemic SFN injection compared to controls, and these changes were associated with increases in heme oxygenase 1. [236] Ping et al. also reported protective effects of SFN in neonatal rats when administered prior to hypoxic ischemia, with effects also mediated by SFN induced activation of Nrf2 and subsequently heme oxygenase 1. [237]

SFN has also been shown to attenuate inflammation. Under physiological conditions, NF-κB is bound to the inhibitor kappa B in the cytoplasm, which prevents translocation of NF-κB to the nucleus. Under stimulating conditions when an inflammatory response is necessary, upstream mechanisms activate the inhibitor kappa B kinase. Activation of inhibitor kappa B kinase leads to phosphorylation of inhibitor kappa B, which results in its dissociation from NF-κB. The inhibitor kappa B is then targeted for degradation and NF-κB is free to translocate to the nucleus. Brain injury can be initiated and sustained by inflammation, via activation of NF-κB. Innamorato et al. injected wild type and Nrf2 -/- knockout mice with LPS and found that the knockout mice had an aggravated inflammatory response compared to wild type. [238] Microglial cells preincubated with SFN (6-10 μM) exhibited an attenuated inflammatory response following LPS exposure. [238] In a mouse model, a 50 mg/kg i.p. injection of SFN induced high levels of Nrf2 and

its downstream phase II enzyme heme oxygenase 1, for up to 2 days. [238] A study by Kraft et al. found that Nrf2 activation in a small population of astrocytes protected neighbouring neurons via alterations in gene expression involved with glutathione, inflammation, and apoptosis, among many others. [239] Bergström et al., found that brief Nrf2 activation by SFN in astrocytes produced downstream phase II enzymes for 24-48 hours. [240]

BrSp may offer some advantages over conventional medicine. BrSp are safe for consumption by pregnant women. [241] Thus, the need to identify a high-risk population (e.g., those more likely to undergo preterm labour) is not required. This is of particular advantage since some women may have asymptomatic infection. In contrast, pharmaceutical interventions require the identification of individuals at risk, as unnecessary drug administration may generate moral and ethical concerns, and may induce unwanted side effects. [242] Cruciferous vegetables are consumed at any point during gestation with no moral or ethical concerns, as they are food. Our laboratory has shown that SFN in pregnant rats fed BrSp transfers across the placental barrier into fetal tissues (n=12 pups from 2 dams/group). [243] In addition, our laboratory has also shown that BrSp consumption during pregnancy and lactation offers neuroprotection, in a model of placental insufficiency in pregnant Long-Evans rats (n=15 litters, 88 pups). [243] Intrauterine growth-restricted offspring from dams that underwent bilateral uterine artery ligation to induce placental insufficiency displayed several neurodevelopmental delays, had decreased hippocampal cell numbers, increased astrocytes in the corpus callosum and cingulum, reduced corpus callosum and cingulum thickness, enlarged ventricular area, and reduced myelin basic protein immunodensity. [243] Consumption of BrSp prevented aberrant reflex outcomes and some aspects of the pathology. [243] In addition, our laboratory has found these findings to permanent in nature,

conferring life-long protection against behavioural deficits (Bahry et al. 2016 – unpublished data). Moreover, BrSp dietary supplementation also appears to confer protection at the level of dendritic arborisation, in association with the prevention of behavioural deficits, compared to those rodents that remain untreated.

1.8. CONCLUSIONS

Chorioamnionitis can result in placental and fetal inflammation, however the precise mechanism remains to be elucidated due to several factors including difficulty identifying the timing of the insult, duration of inflammation, origin of cytokines, and the cytokine profile. [244,245] White matter injury is the hallmark feature of CP, which corresponds to abnormal motor function and incomplete cortical wiring. Gray matter injury corresponds to cognitive and behavioural dysfunction, but has not been as thoroughly investigated as the white matter injury. Further, subtle forms of inflammation not known to cause cystic or diffuse white matter injury, but rather neuronal and glial dysmaturation, are becoming prevalent; however, the mechanisms leading to dysmaturation have yet to be fully elucidated. Therapeutic interventions to protect the developing brain from chorioamnionitis are lacking. Cruciferous vegetables such as BrSp may be a novel prophylactic intervention, offering advantages over conventional drugs. Thus, BrSp may be useful as a preventative measure against fetal brain injury that results from *in utero* inflammation and leads to neurodevelopmental deficits such as CP.

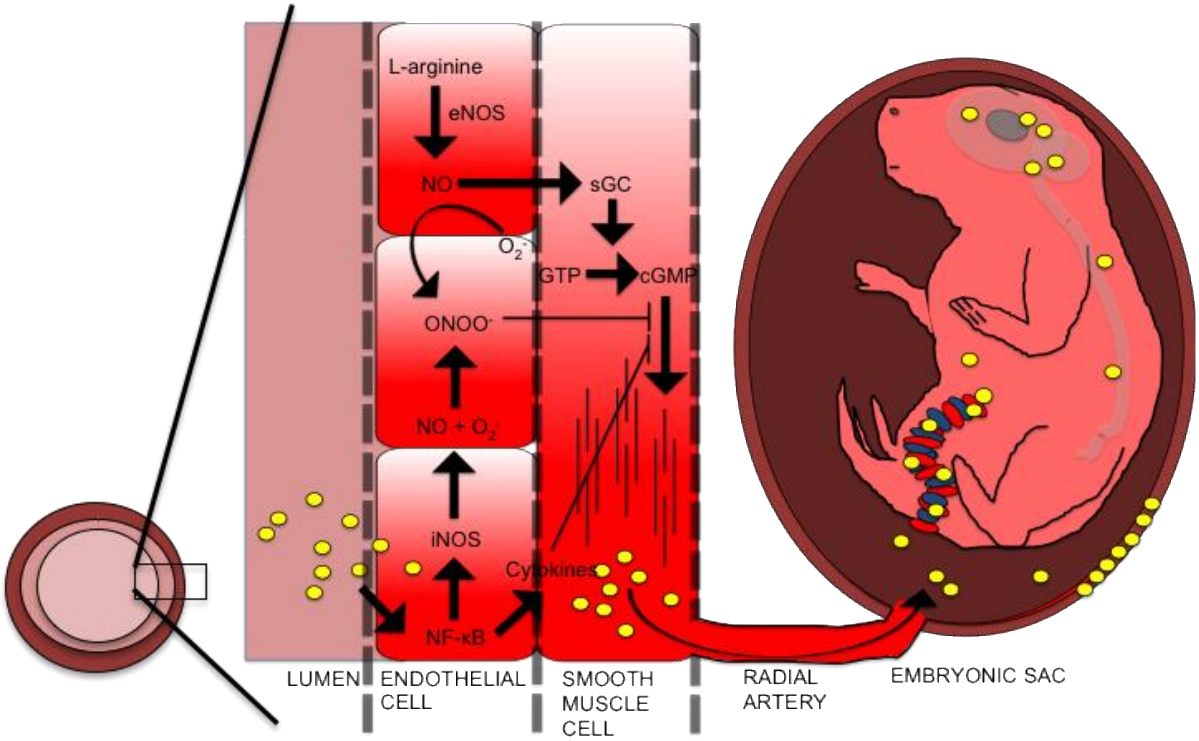


Figure 1.1. Schematic image of the NO-cGMP pathway mediating vasorelaxation in the uterine artery. During pregnancy, the main mediator of vasodilation is NO. NO is converted from L-arginine by endothelial nitric oxide synthase in endothelial cells. NO then diffuses into smooth muscle cells where it activates soluble guanylyl cyclase (sGC). Soluble guanylyl cyclase converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), which induces relaxation of smooth muscle cells. During conditions of inflammation and oxidative stress, cytokines can activate NF-κB, which increases production of cytokines and iNOS in endothelial and vascular smooth muscle cells. Cytokines can gain access to the placenta and enter the fetus. Expression of iNOS increases production of NO, and in combination with O_2^-, forms $ONOO^-$. This inhibits vasorelaxation and promotes vasoconstriction. NO = nitric oxide. sGC = soluble guanylyl cyclase GTP = guanosine triphosphate. cGMP = cyclic guanosine monophosphate. NF-κB = nuclear factor kappa light chain enhancer of activated B cells. iNOS = inducible nitric oxide synthase. O_2^- = superoxide anion. $ONOO^-$ = peroxynitrite

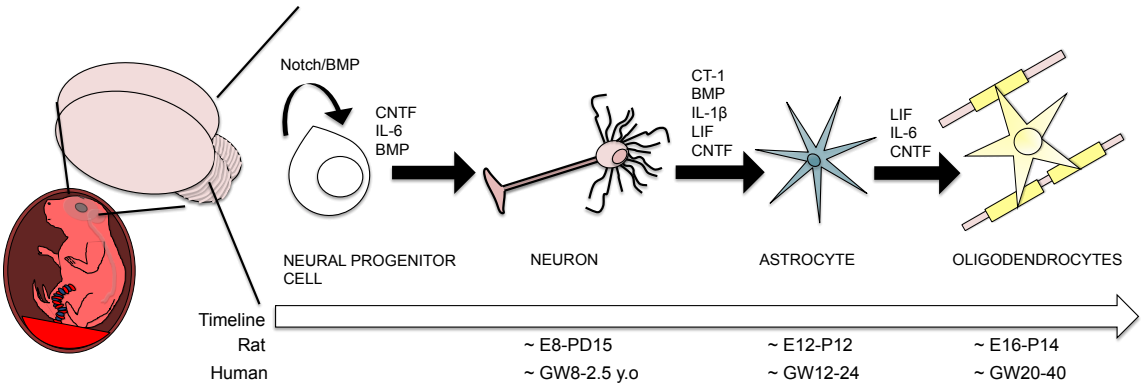


Figure 1.2. The role of neuropoietic cytokines on neuroepithelial cells. [158,193,246-251] A timeline is presented for the occurrence of the proliferative stages in rat and human brains. During development, the fetal brain undergoes successive stages of proliferation, referred to as neurogenesis and gliogenesis. Neurogenesis produces neurons, followed by gliogenesis, which produces astrocytes and then oligodendrocytes. The IL-6 family and gp130 receptors are involved in regulation of these successive (although overlapping) stages. The cytokines act as signaling cues that can promote or inhibit the cell fate of neural progenitor cells. Microglia (not shown) are not produced in this succession because they are the only non-neural cells found in the brain. Furthermore, brain development continues well beyond the time-points presented. BMP = bone morphogenic protein. CT = cardiotrophin-1. IL-1β = interleukin 1β. LIF = leukemia inhibitor factor. CNTF = ciliary neurotrophic factor. IL-6 = interleukin 6. E = embryonic day. GW = gestational week. y.o = years old. Gp130 = glycoprotein 130.

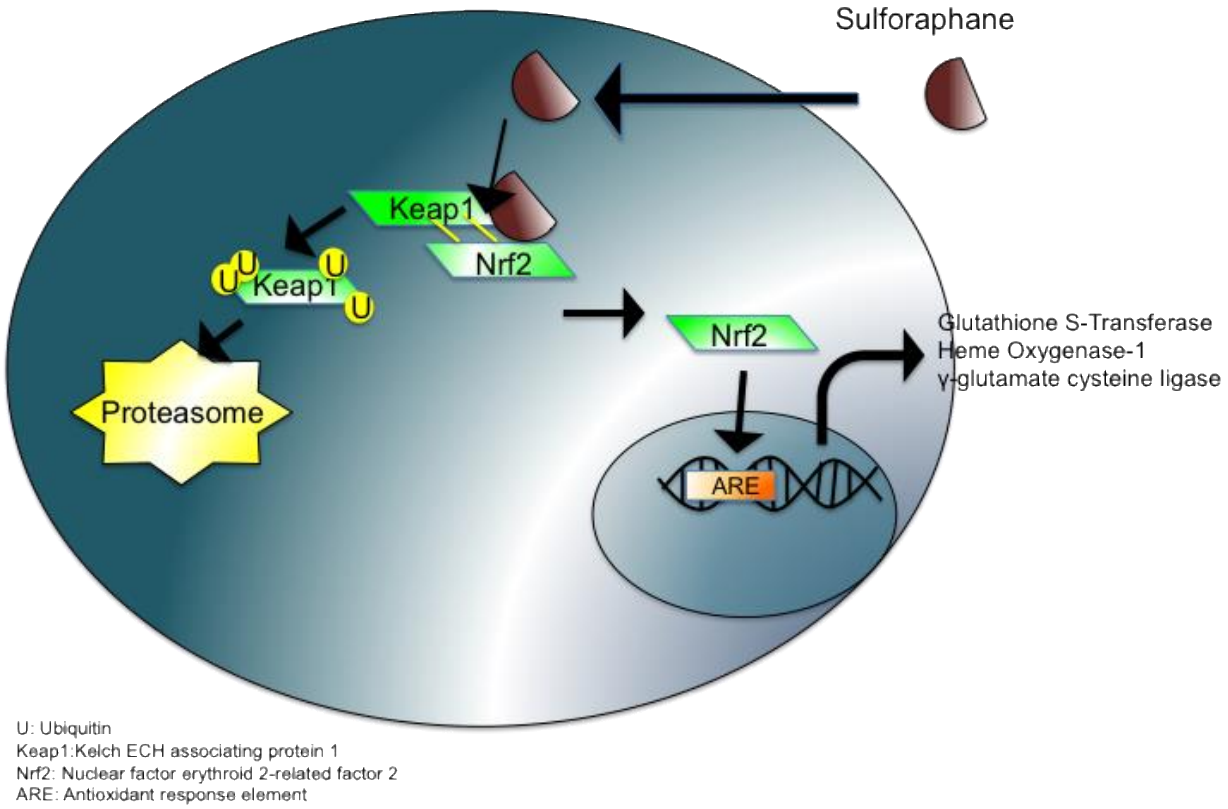


Figure 1.3. SFN mediated upregulation of endogenous phase II enzymes via Keap1/Nrf2. The Keap1/Nrf2 pathway is activated by SFN to provide protection against oxidative stress.[235,240,252,253] Upon breakdown of BrSp, glucoraphanin is converted to SFN by myrosinase. SFN enters the cell and disrupts the Keap1/Nrf2 complex. Once the two signaling molecules are released from one another, Keap1 is targeted for degradation and Nrf2 is free to translocate to the nucleus. Keap1 becomes ubiquitinated and sent to the proteasome. Nrf2 binds to its cognate DNA sequence, the ARE promoter gene. Activation of this promoter gene induces production of endogenous phase II enzymes such as glutathione S-transferase, heme oxygenase-1, and γ-glutamate cysteine ligase. U = ubiquitin. Keap1 = Kelch ECH associating protein 1. Nrf2 = Nuclear factor erythroid 2-related factor 2. ARE = antioxidant response element.

2. OBJECTIVES AND HYPOTHESES

2.1. OVERALL OBJECTIVE

The precise pathophysiology associated with the FIR remains to be fully elucidated and a lack of therapeutic interventions available to protect the developing brain poses a significant problem. The overall objective of the study was to characterize a model of CP induced by inflammation and to test the possible neuroprotective properties of a cruciferous vegetable, BrSp.

2.1.1. Objective I

The first objective of the study was to examine whether newborn offspring exposed to *in utero* inflammation exhibit intrauterine growth restriction, developmental reflex delays, and behavioural deficits (anxiety and exploratory behaviour), and development of white matter injury (periventricular leukomalacia), all of which are characteristics of CP. We aimed to determine if dietary supplementation with BrSp was able to prevent growth restriction, developmental reflex delays, behavioural deficits, and white matter injury.

2.1.2. Objective II

The next objective was to identify changes in placental and cerebral inflammatory cytokines to ascertain the presence of *in utero* and fetal inflammation. The influence of cytokines on neurotrophic factors, which are polypeptides involved in the developmental of the fetal brain,

were also investigated as a downstream target. Changes to levels of neurotrophic factors could alter brain maturation; therefore markers of brain development (neuronal and oligodendroglial maturation, astrogliosis) were also examined. Furthermore, the effects of BrSp on cytokine expression, neurotrophic factors, and fetal brain maturation were subsequently investigated.

2.1.3. Objective III

The FIR has been shown to result in growth restriction. The next objective was to identify whether inflammation altered blood flow and enhanced uterine artery constriction, leading to intrauterine growth restriction. BrSp were also investigated to determine if the intervention was able to prevent growth restriction by restoring blood flow and uterine artery relaxation.

2.2. OVERALL HYPOTHESIS

The overall hypothesis was that the inflammatory model would produce features of CP. Furthermore, it was hypothesized that ingestion of BrSp during late gestation and lactation as a prophylactic intervention would afford protection against inflammation in the offspring such that the abnormalities present in the model would be completely abolished.

2.2.1. Hypothesis I

Our primary hypothesis is that: 1. BrSp dietary supplementation in a model of FIR, will prevent the brain injury, developmental delays and behavioural deficits, associated with a

cerebral palsy phenotype. Specifically we will develop a model of maternal inflammation, caused by LPS that will result in a FIR and induce neurodevelopmental reflex delays and behavioural abnormalities, in rodents. Pathological assessments will reveal a lack of myelination, ventriculomegaly, and reduced brain volume.

2.2.2. Hypothesis II

It was hypothesized that following inflammation, an increase in pro-inflammatory cytokines, pro-oxidants, and decrease in glutathione will occur in the fetal brain, reflecting inflammation and oxidative stress. The inflammation and oxidative stress will then cause downstream alterations to neurotrophic factors expression. This disruption will alter neuronal and oligodendroglial maturation and promotion of activated microglia and astrogliosis. BrSp supplementation was expected to prevent the inflammatory cascade and oxidative stress via activation of the Nrf2 pathway. Prevention of upstream inflammation and oxidative stress was predicted to promote restore proper brain development.

2.2.3. Hypothesis III

Inflammation and oxidative stress will increase vasoconstriction and reduce vasodilation in maternal uterine arteries. These changes would subsequently reduce umbilical blood flow to the fetus resulting in growth restriction. BrSp supplementation during pregnancy was anticipated to rescue uterine endothelial dysfunction, inhibit reductions in blood flow in the uterine and umbilical arteries, and thereby prevent growth restriction.

2.3. SUMMARY

Maternal infection and inflammation is a significant risk factor for the development of CP. CP is a complex neurodevelopmental disorder arising predominantly from insults occurring prior to the time of birth. Thus, the objectives of this study were to identify the behavioural and pathological outcomes in a model of fetal inflammation, and to determine whether BrSp, a cruciferous vegetable, prevent the phenotypic and pathologic consequences of CP.

3. **METHODS**

3.1. ANIMAL MODEL

3.1.1. *Experimental Animals*

The following section describes the method for the experimental model (Figure 3.1). Long-Evans rats were chosen for experiments as they have been shown to have superior motor performances and visual acuity compared with other strains of rats. [254,255] Female and male Long-Evans rats were purchased from Charles River Laboratories at 12 weeks of age. After arrival, rats were placed in conventional housing in the Health Sciences Laboratory Animal Facility. The rats were given 1-2 weeks to acclimatize to the new environment, and were handled to reduce their stress and anxiety. The Animal Care and Use Committee, Health Sciences, at the University of Alberta, approved all animal studies.

3.1.2. *Breeding*

Two female and one male Long-Evans rat were placed in a cage overnight for breeding. The following morning, the females were removed and a vaginal smear was obtained. The solution was analyzed under a light microscope to assess the presence of sperm, which confirmed the first day of pregnancy, embryonic day (E) 1. Dams were randomly allocated into one of four groups: i) Saline, ii) Saline + BrSp, iii) LPS, and iv) LPS + BrSp. Animals were housed under 12 h light/dark cycle (6 am/6 pm), and food and water are available *ad libitum*.

3.1.3. *Endotoxin Administration*

Intrauterine inflammation was induced using a protocol adapted from previous studies.[256-259] Briefly, pregnant dams were given intraperitoneal injections of either 100 µl of saline (vehicle control), or 200 µg/kg of LPS (*E. coli* serotype 0127:B8, Sigma Biochemical, Oakville, ON, Canada) diluted in 100 µl of saline, every 12 hours on E19 and 20, for a total of 4 injections. Changes in body weight and temperature were recorded using a scale and rectal temperature probe (thermometer 4600, Measurement specialties, Ohio) prior to each injection, to monitor the physiological response to LPS. If body weight changed more than 10%, and/or if body temperature fell below 32°C, dams were removed from the study. Long-Evans rats gave birth on E23, which was recorded as the first day of life (PD1).

3.2. BROCCOLI SPROUT PREPARATION AND SUPPLEMENTATION

The BrSp were prepared according to the methods of Wu et al.[230] BrSp (Mumm's Sprouting seeds, Saskatchewan) seeds were soaked in water for 2-3 hours and spread out in a sprouting box. Seeds were watered twice a day. On the fourth day, BrSp were harvested and air-dried at room temperature for seven days. Dams were fed 200 mg of dried BrSp in a ceramic dish beginning on E14 until PD21. This BrSp quantity was chosen based on previous studies showing neuroprotective effects in a model of hypertension and placental insufficiency, as well as for the SFN content, which approximates 5.5 µmoles.[230,231,243] Furthermore, our laboratory identified that consumption of 200 mg of dried BrSp versus a 500 µg SFN intra-peritoneal injection was equivalent to 31.9 ± 4.6 pmol/mg and 27.0 ± 8.3 enzyme activity, respectively, in

the rat fetus (*n*=12 pups/group from 2-3 dams).[243] Throughout the study period, dams that did not consume BrSp two days in a row were removed from the study.[230,231] Overall, only 1 dam from objective 1 was removed.[230,231]

3.3. OBJECTIVE 1 METHODS

3.3.1. Experimental Design

The first objective of the study was to measure developmental reflex delay, behavioural deficits, and periventricular leukomalacia, characteristics present in CP patients, following *in utero* inflammation and administration of BrSp (Figure 3.2). Following birth, rat pups and dam were left alone on PD2 to facilitate maternal bonding. Pups were observed daily to ensure proper feeding and care by the dam. Experimental groups included: 1) Saline (*n* = 7 dams), 2) Saline + BrSp (*n* = 7 dams), 3) LPS (*n* = 7 dams) and 4) LPS + BrSp (*n* = 7). Each litter was culled to 10, when possible.

3.3.2. Tissue Collection

Pups were anesthetized with 5% isoflurane and immediately euthanized by decapitation. Brain tissue was collected on PD21 and stored at -80°C. PD21 brains were divided into two groups. The first group of brains were frozen in isopentane in an ethanol/dry ice bath and stored at -80°C for later histological processing. For the second group, brains were placed in a brain matrix with the ventral side of facing upwards. The tissue anterior to the olfactory bulbs was

removed. The tissue posterior to the brain stem was removed. The remaining tissue was separated into three sections; cortex, corpus callosum, and deep gray matter, and were frozen in isopentane as described above. The tissue was then stored at -80°C for future experiments.

3.3.3. Weights

The weights of the offspring were recorded every morning, between 9 and 10 am, from birth until PD21, to determine if the LPS model resulted in intrauterine growth restriction and if the pups displayed catch up growth. Intrauterine growth restriction was defined as a weight of two standard deviations below the mean weight of healthy pups. [260] Previously, our laboratory recorded the birth weights from four naïve litters (n=56 pups) and determined that the average birth weight was 6.28 ± 0.38 g (mean ± SD), and thus, intrauterine growth restriction was defined as ≤ 5.52 g.

3.3.4. Neurodevelopmental Reflex Testing

Pups underwent a daily series of neurodevelopmental reflex testing from PD3-21, between 9 am and 12 pm. Pups were transported to the laboratory at least one hour prior to testing to allow for acclimatization to the new environment. Pups were then removed from the dams and examined underneath a warm lamp (31°C) to maintain body temperature. Reflex testing began 3 days before known acquisition dates to capture the presence of the reflexes. The first day of appearance of a reflex was recorded when the pup was able to perform it two days in a row. The reflex testing was adapted from Fox WM. [261,262]

Reflex Testing:

Forelimb grasp – A blunt object was stroked against the forepaws of the rat pup. A successful reflex was denoted when the paw grasped the blunt object. This was recorded as 0, 1, or 2 for neither, one, or both successful paw grasps, respectively. Testing began on PD3.

Hindlimb grasp - Both hindpaws of the pups were stroked with a blunt rod and the day the grasping reflex was detected was recorded. This was recorded as 0, 1, or 2, for neither, one, or both successful paw grasps, respectively. Reflex testing began on PD3.

Hindlimb placing – The dorsum of the hindpaw was stroked with the edge of a flat surface. The ability of the affected hindpaw to lift and be placed down on that surface was recorded. This was recorded as 0, 1, or 2, for neither, one or both successful paw grasp, respectively, beginning on PD4.

Righting –Rat pups were placed in a supine position and the ability of the rat pup to flip over with the correct forepaw and hindpaw posture was recorded. This was recorded as time in seconds it took for the pup to right itself, with a maximum of 15 seconds. Righting reflex recording began on PD3.

Cliff avoidance - The forepaws of the rat pup over placed over an edge and the ability of the rat pup to turn away from the 'cliff' was observed. This was recorded as presence or absence of the reflex. Testing began on PD4.

Gait – The pup was placed in the center of a 30 cm diameter filter paper and the ability of the rat pup to move and place both forepaws outside the perimeter of the filter paper was observed, with a maximum of 30 seconds allocated to the task. This was recorded as time in seconds it took for the pup to move outside the filter paper. Reflex testing began on PD6.

Accelerated righting – Pups were dropped, twice daily, in a supine position 12 inches above a foam landing. The ability of the pup to right itself and land on all 4 feet was assessed. This was recorded as 0, 1, or 2, where 0 was recorded when the pup lands on its back, 1 for its side, and 2 for successful righting. The task was performed twice each day until the pup was able to achieve a score of 2 at least two days in a row. Reflex recording began on PD14.

Sensory Maturation:

Auditory startle - A loud noise was applied directly in front of the rat pup to assess the presence of a startle response. This was recorded as either a presence or absence of a startle response. Reflex recording began on PD10.

Posture - The day the rat pup evolved from an immature posture to a mature posture, consisting of hindpaws tucked underneath its body when moving and lifting its abdomen up was assessed. This was recorded as either a presence or absence of a mature posture, beginning on PD12.

Eye opening – The day both eyelids were open and eyes were visible was recorded. This was record as either both eyes open or both eyes not opened. Testing began on PD12.

3.3.5. *Open Field Behaviour*

On PD21, pups were assessed for activity, exploratory, and anxiety behaviour by counting the number of times the pups reared, head-lifted, ambulated, urinated, defecated, and time spent in the center of the open field box, and grooming. Pups were placed in a 44 cm square plexiglass box and videotaped for 5 minutes. Each activity was scored twice by two evaluators blinded to the experimental groups. The following activities were assessed and recorded:

Rearing – The number of times the pups stood on their hindlimbs.

Headlifts – The number of times the pups stretched their necks without lifting their forelimbs off the ground.

Ambulation – The number of squares the pups crossed by moving all four limbs into a new square.

Urination – The number of times the pups urinated.

Defecation – The number of fecal pellets produced by the pups.

Time spent in the center – The amount of time in seconds the pup spent in the middle four squares.

Grooming – The amount of time the pups groomed.

3.3.6. Histology

Brain tissue was collected on PD21 for hematoxylin and eosin and immunohistochemistry staining (Table 3.1). Fresh frozen brains were sectioned using a cryostat (Reichert-Jung Cryocut 1800) to obtain ten 14 μm coronal sections. Sections were collected every 0.5 mm beginning from where the corpus callosum first joins together. Sections were placed on a slide, and stored at -20°C until further processing.

Sections were stained with hematoxylin and eosin for volumetric and gross anatomical analyses. Sections were pre-fixed with formalin, rinsed with water, dehydrated (80%, 95%, and 100% ethanol), cleared (xylene), and rehydrated (100%, 95%, 80% ethanol and water). Slides were submerged in a Harris hematoxylin staining, rinsed with water, dipped in acid alcohol, and immerse in tap water to facilitate colour transition from purple to blue. Slides were then dipped in Eosin, washed with water, dehydrated, cleared with xylene, and coverslipped with cytoseal (Thermo Scientific).

Sections were immunostained with the following primary antibodies: myelin basic protein (1/1000, Covance), and anti-Olig2 (1/3000, Millipore) and anti-fractin (1/1500, # Millipore), which are a pan-oligodendroglial cell and axonal apoptotic protein markers, respectively. [33] Sections stained for myelin basic protein were immersed in precooled methanol (-20°C) for 10 minutes, and then washed in physiological buffer solution (PBS, 0.05 M) followed

by PBS/0.1% Triton. The sections were blocked with 20% normal horse serum in 0.1% PBS/Triton and incubated overnight at 4°C with the myelin basic protein. The next day, myelin basic protein slides were washed with PBS/0.1% Triton, immersed in 0.3% hydrogen peroxide in 50% methanol to inhibit any endogenous peroxidase activity prior to washing with PBS. After PBS washes, all slides were incubated with secondary antibody (biotinylated rat adsorbed horse anti-mouse IgG, 1/200, Vector, or biotinylated anti-rabbit IgG (H+L), 1/400, Vector) for one hour. Slides were washed with PBS/0.1% Triton, incubated with avidin-biotinylated enzyme complex (#PK-6100 vectastain ABC kit, Vector Laboratories), washed with PBS/0.1% Triton, and stained with 3,3-diaminobenzidine (#SK-4100 peroxidase substrate kit, Vector laboratories). Once sections reacted, the slides were rinsed with PBS to remove excess 3,3-diaminobenzidine and coverslipped with aqueous mounting media. A negative control was included in every staining procedure.

Sections designated for anti-Olig2 and anti-fractin were prefixed in 10% normal buffer formalin, washed with water, dehydrated, clear, and rehydrated. Slides were then washed with PBS, incubated with 0.3% hydrogen peroxide in 50% methanol, washed with PBS and PBS/0.1% Triton, and blocked for one hour with normal goat serum. Olig2 and Fractin slides were immediately placed into PBS. After PBS washes, all slides were incubated with the secondary antibody (biotinylated rat adsorbed horse anti-mouse IgG, 1/200, Vector, or biotinylated anti-rabbit IgG (H+L), 1/400, Vector) for one hour. Slides were washed with PBS/0.1% Triton, incubated with avidin-biotinylated enzyme complex, washed with PBS/0.1% Triton, and stained with 3,3-diaminobenzidine. Once sections reacted, the slides were rinsed with PBS to remove excess 3,3-diaminobenzidine, washed in water, and dehydrated. Slides were

cleared with xylene and coverslips were mounted with cytoseal (for Olig2 and Fractin). A negative control, which was not incubated with primary antibody, included in every staining procedure. Stained slides were imaged with Leica LAS EZ programs using a light microscope (Leica DM 2000 LED). Brain and ventricular volume, cell counts, and densitometry were accomplished using ImageJ software.[28]

To assess brain and ventricular volume, serial sections 0.5 mm apart were obtained and stained with hematoxylin and eosin. The location where the anterior commissures connected was referred to as bregma (Figure 3.3). Two sections before bregma, bregma, and five sections posterior were used to calculate volume using Cavalieri's Principle, integrating distance between and area within sections. To determine cell counts and densitometry, one anterior section (0.5 mm) and one posterior to bregma (3.0-3.5 mm) were used. The corpus callosum next to the midline and the center of the cingulum were used to count Olig2 cells and calculate myelin basic protein density at 40X.

3.4. OBJECTIVE 2 METHODS

3.4.1. Experimental Design

Our next objective was to determine cytokine and oxidative stress induced alterations in neuronal maturation and glial activation. Cytokine and neurotrophic factor mRNA expression were evaluated on E19 and E22, followed by inflammation, oxidative stress, cell death, and neurodevelopmental protein markers on PD1, PD7, and PD21 (Figure 3.4).

3.4.2. Tissue Collection and Preparation

On E19 and E22, dams were anesthetized with isoflurane. Once the dam was heavily sedated, a laparotomy was performed. 1 mL of blood was collected in a 1 mL syringe coated with 0.5 M ethylenediaminetetraacetic acid (1.5-2 mg/ml), centrifuged at 5000*g* for 10 mins, and plasma collected and stored at -80°C. Maternal liver, kidneys, uterine artery, mesenteric artery, abdominal aorta, thoracic aorta, placentas, and fetal brains and livers were collected and stored at -80°C. Extracted tissue was frozen immediately in isopentane in a dry ice ethanol bath. In a separate group of animals, pups were anesthetized with isoflurane until heavily sedated and brains were removed at PD1, 7, and 21 (as mentioned in objective 1) for matrix metalloproteinase (MMP) analyses. PD1 rat pups were randomly selected and perfused with 10 mL ice-cold saline at a rate of 500 µl/min. These brains were perfused to remove any contaminants of blood, which contains MMP-2. Brain tissue (irrespective of age) was homogenized in Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA, #78510) containing both protease and phosphatase inhibitors (Thermo Scientific, Pierce, Rockford, IL, USA, #88669). The homogenates were centrifuged at 10 000 x g for 10 minutes and the supernatant was collected. The protein concentration was determined using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, #23225, Thermo Scientific).

3.4.3. Nuclear Extraction

Nuclear extracts were collected from PD1 hemispheres for Nrf2 staining according to Baghirova et al. [263] Briefly, brain tissue was homogenized and underwent several steps of

centrifugation and resuspension, until cytoplasmic, membrane, and nuclear extracts were collected. [263] The purity of the nuclear extract was assessed by analyzing the sample with the nuclear histone H3 antibody (1:1000, #9715, Cell Signaling).

3.4.4. Reverse Transcription Quantitative Real Time Polymerase Chain Reaction

IL-6, TNF-α, IL-1β, IL-10, BDNF, NGF, and NT-3 mRNA expression was evaluated in placenta and fetal brains (Table 3.2). Briefly, placenta or brain tissue was ground under liquid nitrogen into a powder. The tissue was stored at -80°C. Once all the tissue was processed, Trizol (Life technologies Cat # 15596-018) was used to isolate RNA according to the manufacturer's instructions. The RNA solution was stored in -80°C until further processing. Once all of the RNA was extracted, the integrity of the RNA (5µl of RNA, 8 µl Tris-EDTA, and 3 µl 5X loading buffer) was assessed on an agarose gel (1.5%) with ethidium bromide (3 µl). Samples were electrophoresed for one-two hours at 100V and subsequently analyzed using a gel imager (BioRad Gel Doc EZ Imager). The integrity was determined by the ratio of 28S ribosomal RNA to 18S ribosomal RNA, with a minimum ratio of 2:1 respectively (Figure 3.5). The RNA concentration was determined using a spectrophotometer set at 260 nm. An absorbance of 1 at 260 nM is equivalent to 40 µg/ml RNA. The concentration of RNA was calculated.

RNA was converted to cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Cat no. 11754-010) according to manufacturer's instructions. The solution was incubated for 10 minutes at 25°C, 60 minutes at 42°C, and 5 minutes at 85°C. The stock solution contained 625 ng of cDNA in the working solution, which was stored at -20°C, and was 2.5

ng/µl. Primers were prepared by adding 150 µl of Tris-EDTA and 475 µl of 10 mM Tris-Cl pH 8.0. Working stocks of 0.5 mL containing 10 pmol/µl primer (5000 pmol of primer) stored at -20°C. A 384-well plate was used to load cDNA samples (2 µl) and SYBR reaction solution containing the primers (2.5 µl of SyBr Dye, 0.25 µl forward primer, 0.25 µl reverse primer). The plate was placed into a LightCycler 480 and underwent pre-incubation, amplification, melting curve, and cooling cycles. Data was collected for melting point and crossing points. Hydrolysis probes were also used to detect mRNA of interest, and the following protocol was used: 1 UNG cycle, 1 denaturation cycle, 50 amplification cycles, and 1 cooling cycle. LightCycler480 software was used to generate the crossing points and fold differences for each gene normalized to the reference gene YWHAZ using the ΔΔct method. Genes using the SYBR reaction solution were assayed in triplicate whereas Hydrolysis probes were in duplicate.

3.4.5. Cytokine Protein Analysis

Cytokine protein concentrations (IL-1β, TNF-α, IL-6, and IL-10) in PD1 brains were detected using the MSD Multi-Spot Assay System proinflammatory kit. The procedure was carried out based on manufacturer's instructions. Protein expression of the pro-inflammatory cytokines was normalized to IL-10 to assess changes to cytokine balance. The ratio was used because proteins lead to the physiological outcomes. An upregulation of both pro-inflammatory cytokine and anti-inflammatory cytokine suggests no changes to the balance whereas increases or decreases in one without change in the other suggest upregulation or downregulation, respectively.

3.4.6. Glutathione Analysis

The ratio of reduced to oxidized glutathione was measured to assess presence of oxidative stress and the availability of reduced glutathione, potentially a result of activated Nrf2 by BrSp. Glutathione was detected using a glutathione assay kit (Cayman Chemical, catalog #703002) according to manufacturer's instructions.

3.4.7. Gelatin Zymography

Perfused PD1 brain tissue homogenates were used for analysis of MMP-2 activity with gelatin zymography.[264-266] A non-heated sample of 20 μg of total protein was prepared and loaded into each well on an 8% polyacrylamide gel infused with gelatin. HT1080 cell conditioned media was used as a positive control and to assure the presence of bands at the same molecular weight. Gels were run for 2 hours at 110V followed by three washes (2.5% Triton X-100) for 20 minutes each to ensure complete removal of excess sodium dodecyl sulphate. The gel was then incubated overnight at 37°C in an incubation buffer (50mM Tris base, 5mM $CaCl_2$ – $2H_2O$, 150mM NaCl, 0.05% NaN_3, pH to 7.6, in a total volume of 250 mL). The next day, the gel was stained with 0.05% Coomassie Brillian Blue G-250 (Sigma) for 2-3 hours. The staining solution consisted of Coomassie Brilliant Blue G-250, 125 mL methanol, 50 ml glacial acetic acid, and 325 ml ddH_2O. Excess Coomassie Blue was removed with an aqueous 4% methanol:8% acetic acid solution. The gel was scanned (GS-800 Calibrated Densitometer scanner and QuantityOne software, BioRad) and samples were quantified with densitometry (normalized to HT1080) with ImageJ.

3.4.8. Immunohistochemistry and Immunofluorescence

The same protocol was followed as in objective 1 for histology (Table 3.1). The sections were also immunostained with the following primary antibodies: Activated microglia CD68 (1/1000, #MCA341R, AbD Serotec), cleaved caspase3 (1/200, #9664L, Cell Signal), Fluoro-Jade B (#LV1378395, Chemicon), and NeuN (1/1600, #MAB377, Millipore). These antibodies allowed for assessment of activated microglia, apoptotic cell death, degenerating neurons, and mature neurons. All antibodies were examined on PD1 brains with the exception of NeuN, which was stained on PD21. The secondary antibodies used were rat adsorbed horse anti-mouse IgG (1/200, #BA-2001, Vector) and biotinylated anti-rabbit IgG (H+L) (1/400, #BA-1000, Vector).

Fluoro-Jade B is a fluorescent stain for degenerating neurons. A 0.001% Fluoro-Jade B working solution was prepared from a 0.1% Fluoro-Jade B stock solution. Briefly, sections were prefixed with normal buffer formalin, washed with water, dehydrated, and immersed in 0.06% potassium permanganate for 15 minutes with agitation. Slides were washed and incubated with 0.001% Fluoro-Jade B working solution for 30 minutes in a dark room. Slides were washed, dried, cleared with xylene, mounted with DPX, and stored in a dark room. Sections were imaged with a fluorescence microscopy (Leica DMIRE2 and CTRMIC) and analyzed with ImageJ.

3.4.9. Immunoblotting

A total of 40 μg of protein was used for both nuclear and hemisphere homogenates. Four times SDS PAGE sample buffer with fresh 2-mercaptoethanol was added to the protein sample,

vortexed, centrifuged, and boiled at 95°C for 5 minutes to denature proteins. A ladder (#161-0373, BioRad) and 40 µl of sample were loaded into a 10-well 4-20% gradient gel (#456-1094, BioRad). The gel was transferred to polyvinylidene fluoride membrane pre-activated for 2 hours at 100V in a Mini Protean Gel Transfer System (BioRad) at 4°C. The membrane was blocked with 5% milk in 0.1% tris buffered saline-tween (or 5% bovine serum albumin) for one hour. Primary antibodies were diluted in the blocking solution and incubated overnight on a shaker at 4°C. The following primary antibodies were used to detect 1) changes in maturation of neurons; growth associated protein 43 (1µg/ml, #ab16053, Abcam), synatophysin (1/200 ab14692, Abcam), syntaxin (1µg/ml, #ab41453, Abcam); 2) oligodendroglial cells (Olig2 1:2500 #AB9610, Millipore), 2',3'-cyclic-nucleotide 3'-phosphodiesterease (5µg/ml, #ab6319, Abcam); and 3) astrogliosis via glial fibrillary acidic protein (1:200, #Z 0334, DakoCytomation) (Table 3.3). Nuclear Nrf2 (1 µg/ml, #MAB3295, R&D Systems) was also evaluated to determine if this was the mechanism of protection afforded by the BrSp. The loading control was β-actin (1:1000, #ab8226, Abcam). The membrane was washed with 0.1% tris buffered saline-tween three times for ten minutes, blocked with secondary antibody (ECL Sheep Anti-Mouse IgG Horseradish Peroxidase-Linked Species Specific Whole Antibody, #NA931, GE Healthcare and ECL Donkey Anti-rabbit IgG Horseradish Peroxidase-Linked Species-Specific Whole Antibody, #NA934, GE Healthcare) at room temperature for two hours, and washed again three times for ten minutes each. Enhanced chemiluminescence (Luminata Classico Western HRP Substrate, #WBLUC0500, Millipore) was used to visualize the bands. The bands were visualized with the G:Box Chemi-XR5 (Syngene) and analyzed with GeneTools software (Syngene). Samples were quantified using densitometry and normalized to the loading control.

3.5. OBJECTIVE 3 METHODS

3.5.1. Experimental Design

Significant growth restriction was observed in fetuses exposed to LPS. This led to the next objective of the study, which involved investigation of uterine and umbilical arteries (Figure 3.6). We assessed the *in vitro* vasoactivity of isolated uterine arteries following *in vivo* exposure to LPS. Impedance to blood flow in the uterine and umbilical vessels was also assessed.

3.5.2. Tissue Collection

At E22, the dam was euthanized and tissue was collected (as discussed in objective 2). Uterine arteries were removed and immediately placed in ice cold physiological saline solution (HEPES 10mmol/l, glucose 5.5 mmol/l, $CaCl_2$ 1.56 mmol/l, KCl 4.7 mmol/l, NaCl 142 mmol/l, $MgSO_4$ mmol/l, KH_2PO_4 1.18 mmol/l, pH 7.4) made fresh prior to tissue collection.

3.5.3. Ultrasound Imaging

On E21, dams underwent Doppler imaging to assess blood flow of both uterine and umbilical arteries. Briefly, dams were anesthetized with 3.5% isoflurane and maintained at 2.5%. Abdominal hair was extracted using both a razor and chemical hair remover to prevent interference when imaging with the ultrasound biomicroscope (model Vevo 2100, VisualSonics, Toronto, ON, Canada). Once the hair was removed, pre-warmed ultrasound gel was applied to the abdomen to facilitate transcutaneous imaging. The dams' heart rate, respiratory rate, and

body temperature were monitored for the duration of imaging. Uterine and umbilical artery's Doppler waveforms were imaged and recorded using a transducer probe at an angle that did not exceed 30°. Images from the arteries were obtained within a one-hour time window. Following image collection, dams were removed and placed back in their cage. Analyses were performed using Vevo 2100 (VisualSonics) imaging software. Doppler waveforms from one uterine artery and three umbilical arteries were used to calculate peak systolic velocity and end diastolic velocity. Three waveforms from each artery were measured and averaged. Waveforms that overlapped with the breathing cycle were excluded. Pulsatility index (PI) and resistive index (RI) were measured using the following equations: PI=(PSV-MDV)/MV and RI = (PSV-MDV)/PSV, where PSV = peak systolic velocity, MDV = minimum diastolic velocity, and MV = mean velocity.

3.5.4. Wire Myography

Uterine arteries were placed in a petri dish filled with ice-cold physiological saline solution. Under a dissecting light microscope, fat and connective tissue was removed. Uterine arteries were sectioned into 2 mm segments, wired with two 40 μm tungsten wires, and mounted onto the baths of the wire myograph (610M, Danish Myo Technology, Aarhus, Denmark) filled with warm (37°C) physiological saline solution. The uterine arteries were normalized to the optimal resting tension, and were equilibrated for 20 minutes. Vessel integrity (both endothelium and smooth muscle) was tested with two applications of phenylephrine ($1x10^{-5}$ M, Sigma) separated by a wash, and a relaxation dose of methacholine ($3x10^{-6}$ M, Sigma) after the second phenylephrine. The vessels were washed and incubated with the following inhibitors for 30

minutes: no drug (control), 1400W (inducible nitric oxide synthase inhibitor) ($1x10^{-6}$ M, #W4262, Sigma), polyethylene glycol superoxide dismutase (peg-SOD) (50 units/ml, #S9549, Sigma), and L-N^G-Nitroarginine methyl ester (L-NAME, pan-NOS inhibitor) ($1x10^{-4}$ M, #N5751, Sigma). The control bath, which did not have inhibitors or free radical scavenger added, allowed for assessment of the arterial responses to phenylephrine and methacholine without any external influences. 1400W inhibits iNOS by binding to the enzyme inducible nitric oxide synthase. Peg-SOD scavenges free superoxide anion, thereby reducing oxidative stress and increasing the bioavailability of NO. L-NAME inhibits production of NO, thereby reducing the ability of NO to mediate vasodilation. A dose response to phenylephrine ($1x10^{-8}$ – $1x10^{-5}$ M) was performed to assess vasoconstrictive capacity and produce a cumulative concentration response curve. Afterwards, baths were flushed and fresh physiological saline solution was added to the baths along with the inhibitor and scavenger for 30 minutes. The dose of phenylephrine that generated 80% maximum constriction was calculated and was used to preconstrict the vessels for ten minutes. An endothelial-dependent relaxation was performed using methacholine, to generate a dose response curve ($1x10^{-10}$ – $1x10^{-4}$ M). Afterwards, a washout period was carried out followed by 30 minutes equilibration with no additional inhibitors. An endothelial-independent relaxation curve was performed using sodium nitroprusside ($1x10^{-11}$ – $1x10^{-5}$ M). The arteries were washed and left for ten minutes. At the end of the experiment, arteries were washed and a high potassium chloride solution was added to induce a final total constriction response. This was performed to eliminate the possible role of non-receptor mediated contraction. A phenylephrine and methacholine cumulative concentration response curve produced for each artery with or without its inhibitors was analyzed for potency (pEC_{50}) and efficacy (E_{max}). The

pEC_{50} is the log of the dose required to elicit 50% of the maximal response whereas the E_{max} is the maximal response of the arteries.

3.6. STATISTICS

To prevent litter bias when using multiparous models, [267,268] pups in the same litter were averaged so that each litter (or dam) represented an $n = 1$. Both LPS and BrSp treatment were delivered through the dam; therefore, the dam represented the experimental unit. Furthermore, pups from the same litter behave more similarly compared to pups from a different litter due to their genetic makeup, nutritional availability, and *in utero* environment. [268]

STATA and Graphpad Prism were used to perform all statistical analyses. Power and sample size analyses were performed to ensure adequate sample size with sufficient power ($\beta=0.80$) to detect significant differences. Two-way ANOVA and Tukey's post hoc test were used to analyze the data because two variables were involved, LPS and BrSp. If a main effect of Sex was detected, males and females were analyzed separately. Significant effects detected by two-way ANOVA are presented as main effect of treatment (LPS or Saline) and diet (BrSp or no BrSp) and/or interaction effect of treatment and diet. The pulsatility index and resistance index were analyzed using a Student T-test. Data are expressed as mean ± SEM. Significance was identified as $p \leq 0.05$. a = significant main effect of Treatment. b = significant main effect of Diet. c = significant interaction effect. * = significant difference between Saline and LPS following Tukey's post hoc test. # = significant difference between LPS and LPS + BrSp

following Tukey's post hoc test. $ = significant difference between Saline and Saline + BrSp following Tukey's post hoc test.

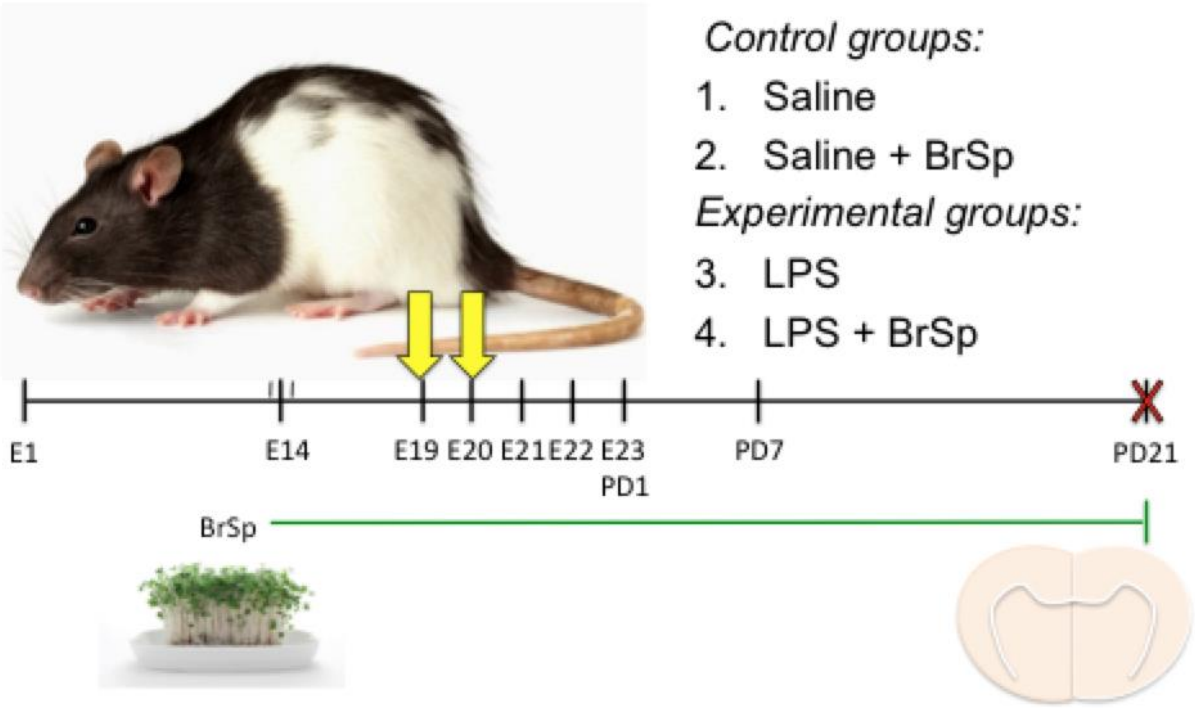


Figure 3.1. The experimental model used to investigate *in utero* inflammation and the protective properties of BrSp. The first day of pregnancy was defined as embryonic (E) day 1. On E14, dams were randomly selected to receive BrSp in addition to their diet. On E19 and E20, dams were randomly selected to receive either Saline (control) or LPS (treatment) i.p. injections every 12 hours over a period of two days. Pups were born on E23, which was also denoted as postnatal day (PD) 1. Pups underwent reflex testing, and were euthanized at several time points to carry out the experiments.

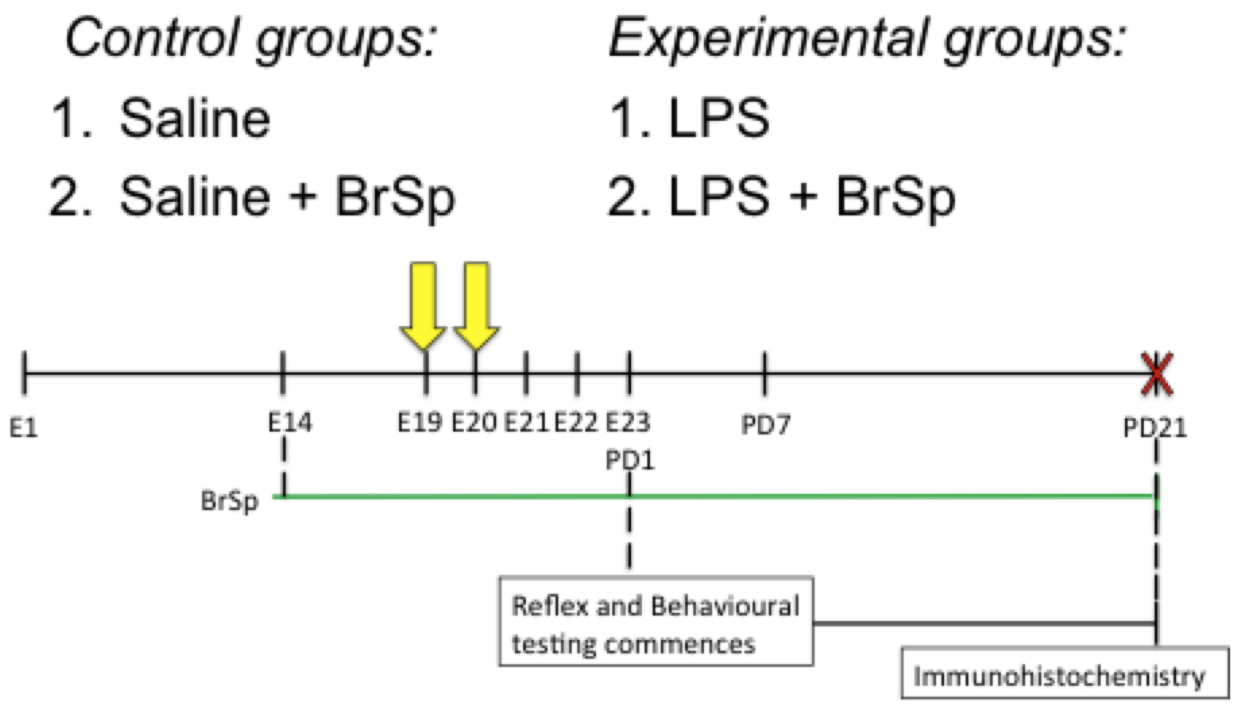
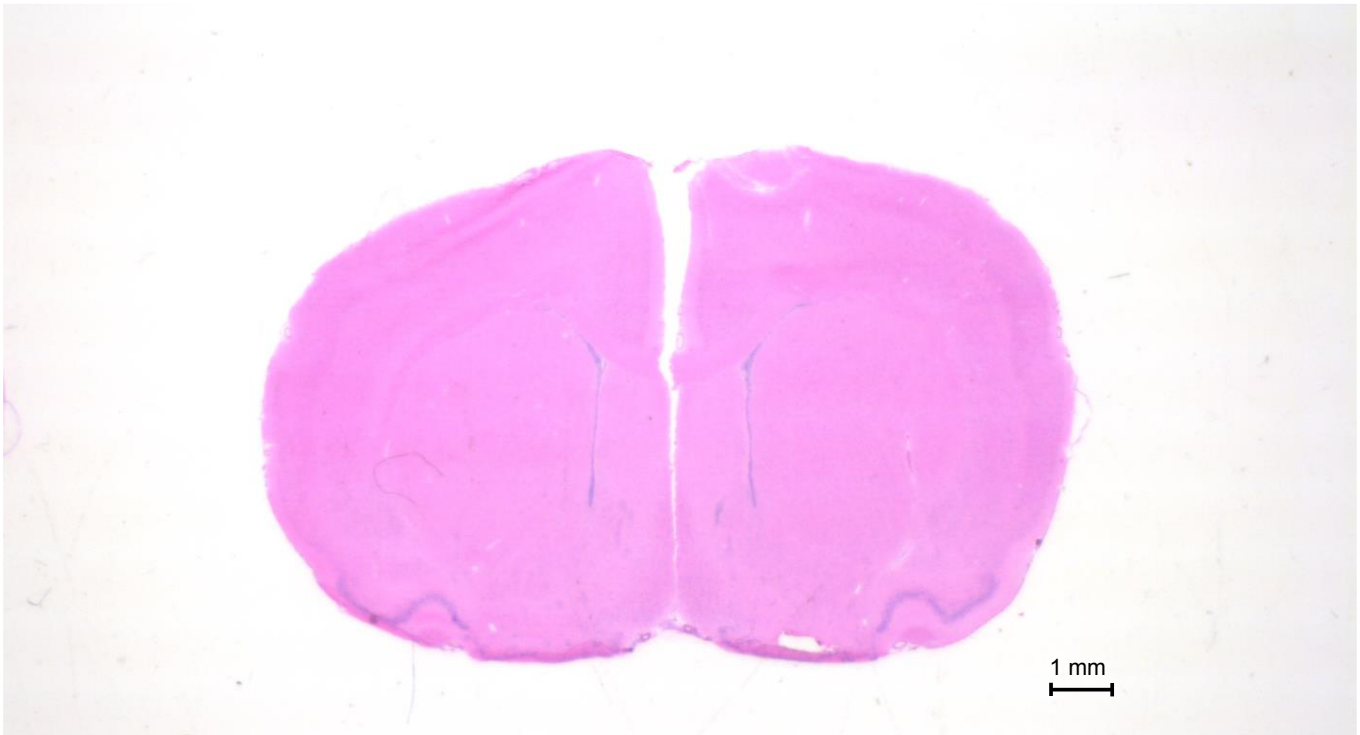


Figure 3.2. Methodological timeline for objective 1.The first day of pregnancy was defined as embryonic (E) day 1. On E14, dams were randomly selected to receive BrSp in addition to their diet. On E19 and E20, dams were randomly selected to receive either Saline (control) or LPS (treatment) i.p. injections every 12 hours over a period of two days. Pups were born on E23, which was also denoted as postnatal day (PD) 1. Beginning on PD3, pups underwent a series of neurodevelopmental reflex testing. On PD21, pups were behaviourally assessed using the open field test. Pups were then anaesthetized and euthanized for brain pathology assessments.

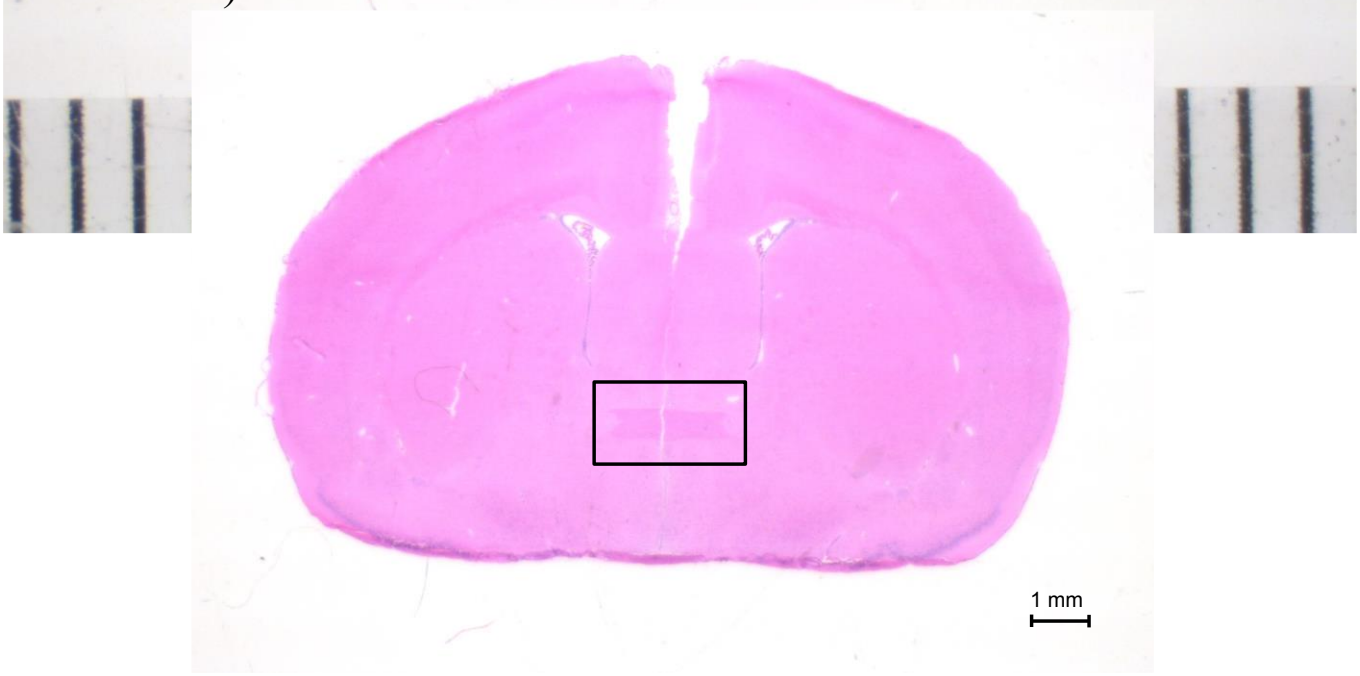
Table 3.1. Antibodies used for immunohistochemistry.

Postnatal Age of Assessment	Antibodies	Concentration	Host	Catalogue Number	Company
PD1	CD68	1/1000	Mouse, monoclonal	MCA341R	AbD Serotec
PD1	Cleaved caspase 3	1/200	Rabbit, polyclonal	9661	Cell Signal
PD21	Olig2	1/3000	Rabbit, polyclonal	AB9610	Millipore
PD21	Myelin Basic Protein	1/1000	Mouse, monoclonal	SMI-94R	Covance
PD21	Fractin	1/500	Rabbit, polyclonal	AB3150	Millipore
PD21	NeuN	1/1600	Mouse, monoclonal	MAB377	Millipore

Table 3.1. Antibodies used for immunohistochemistry. CD68 was used to detect activated microglia. Cleaved caspase 3 was used to detect apoptotic cell death. Olig2 was used as a pan-oligodendroglial stain. Myelin basic protein was used to detect myelin. Fractin was used for detection of apoptotic axons. NeuN was a marker used to analyze mature neurons.



B)



C)

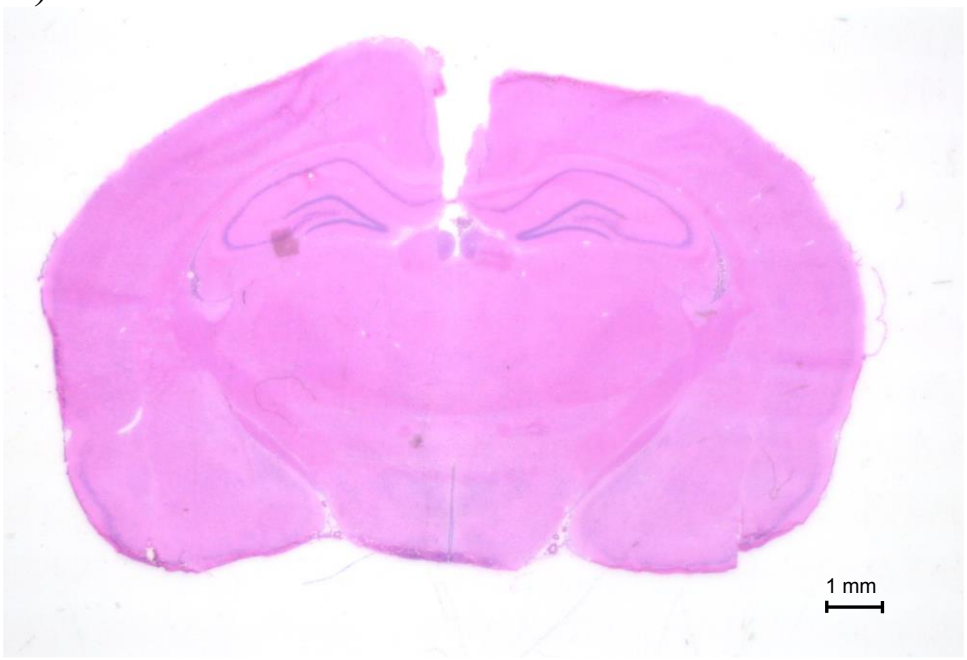


Figure 3.3. Representative images of postnatal brain sections stained with hematoxylin and eosin, used for volumetric analyses. Presented above are anterior (A), bregma (B), and posterior (C) sections of a postnatal day 21 rat brain. Bregma is defined as the section where the anterior commissures join the two hemispheres (outlined in a box in image (B)).

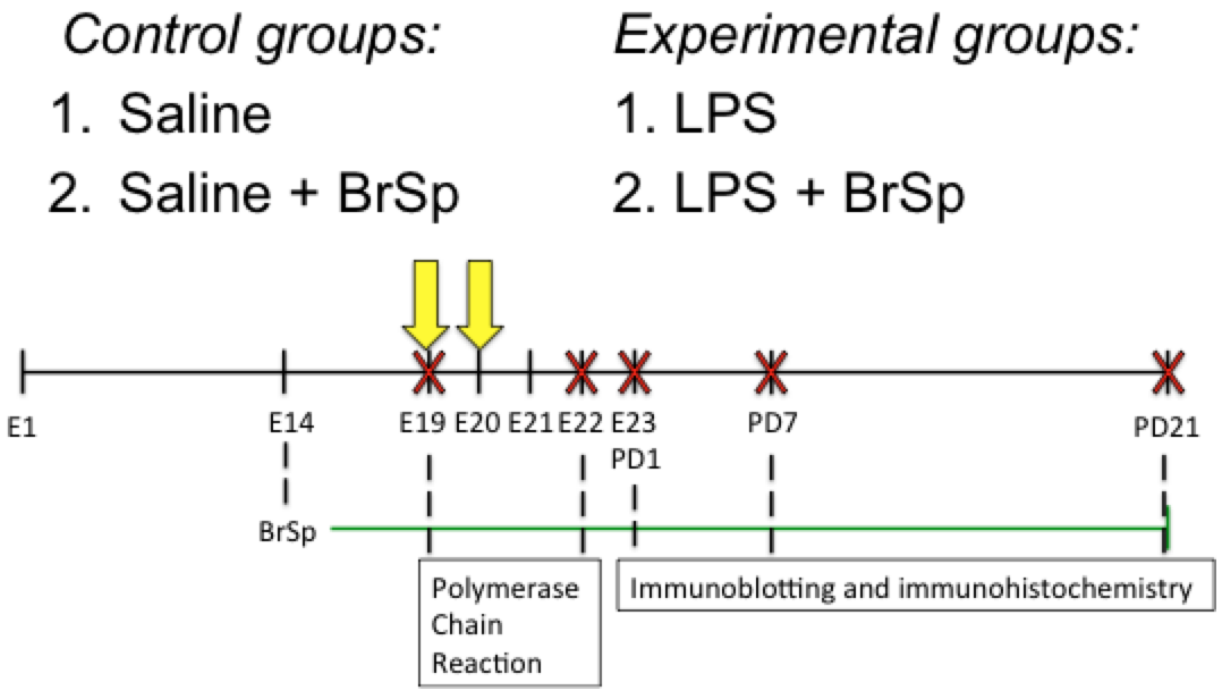


Figure 3.4. Methodological timeline for objective 2.The first day of pregnancy was defined as embryonic (E) day 1. On E14, dams were randomly selected to receive BrSp in addition to their diet. On E19 and E20, dams were randomly selected to receive either Saline (control) or LPS (treatment) i.p. injections every 12 hours over a period of two days. Pups were born on E23, which was also denoted as postnatal day (PD) 1. On E19 and E20, maternal blood, liver, kidney, mesenteric arteries, uterine arteries, and aortas were collected, frozen, and stored at -80°C. Placentas, and fetal brains and livers were also collected on E19 and E20 for subsequent RNA analyses. Brains from PD1, PD7, and PD21 offspring were collected for protein analyses and immunohistochemistry.

Table 3.2 Primers used for polymerase chain reaction experiments to detect mRNA levels of target genes.

A)

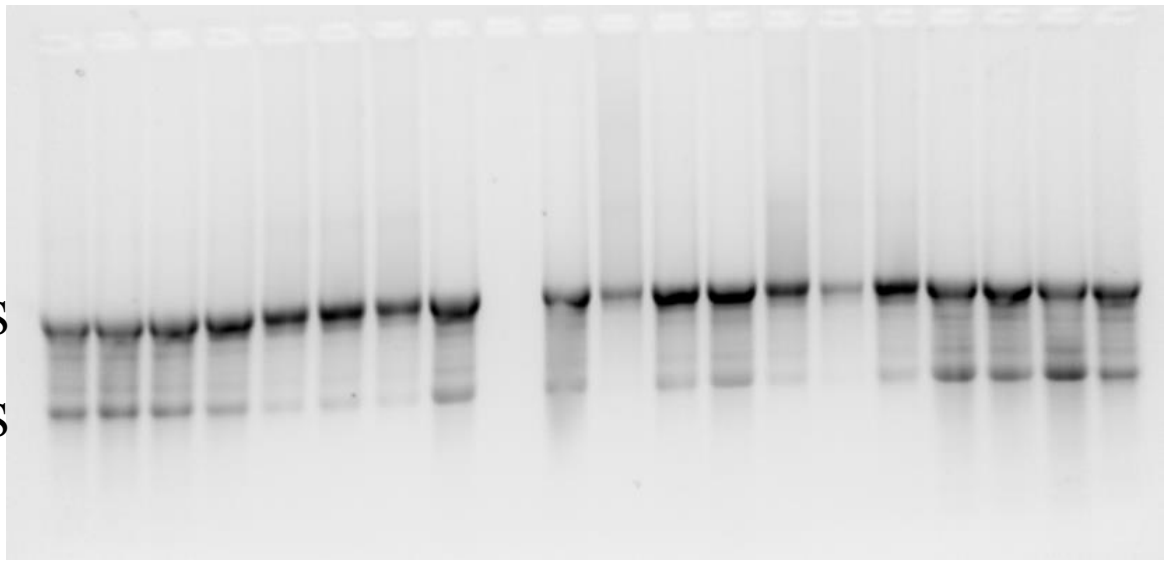
Gene	**Sequence (5'-3')**	**Exon**
Cytokines		
IL1 β	F-CAACTGTGAAATAGCAGCTTTCG	2
	R-CTCTTGTCGAGATGCTGCTG	3
Neurotrophic Factors		
BDNF	F-TTTGTGTGGACCCTGAGTTC	1
	R-AGCCTTCATGCAACCGAAG	2
NGF	F-CCACTCTGAGGTGCATAGC	1
	R-CTGGGACATTGCTATCTGTGTAC	2
NT-3	F-TTCTGCCACGATCTTACAGG	2
	R-TTGATCCATGTTGTTGCCTTG	3

B)

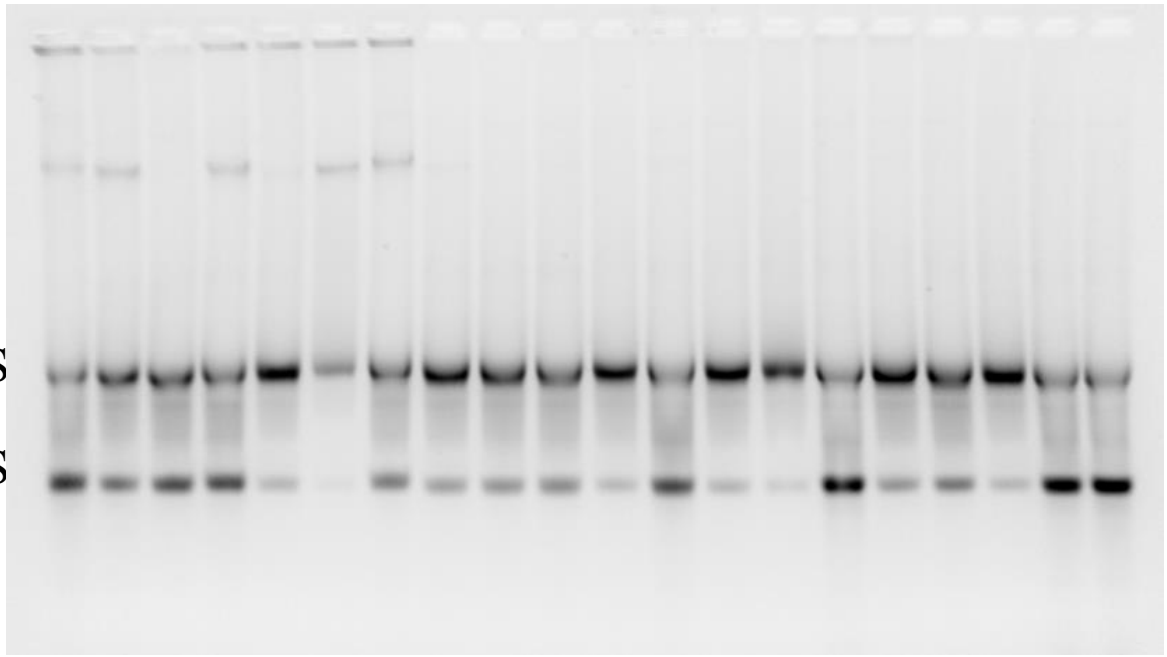
Gene	**Assay ID**	**Exon**
Cytokines		
IL1 β	Rn00580432_m1	5,6
IL6	Rn01410330_m1	3,4
TNF α	Rn01525860_g1	2,3
IL10	Rn01483988_g1	2,3
Reference Gene		
YWHAZ	Rn00755072_m1	2,3

Table 3.2 Primers used for polymerase chain reaction experiments to detect mRNA levels of target genes. Primers were generated and purchased from Integrated DNA Technologies (A) and Hydrolysis probes (B) for mRNA analyses were purchased from Thermo Scientific. The reference gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) was used in the experiments, which is involved with regulating signaling transduction pathways. Bp = base pairs.

A)



B)



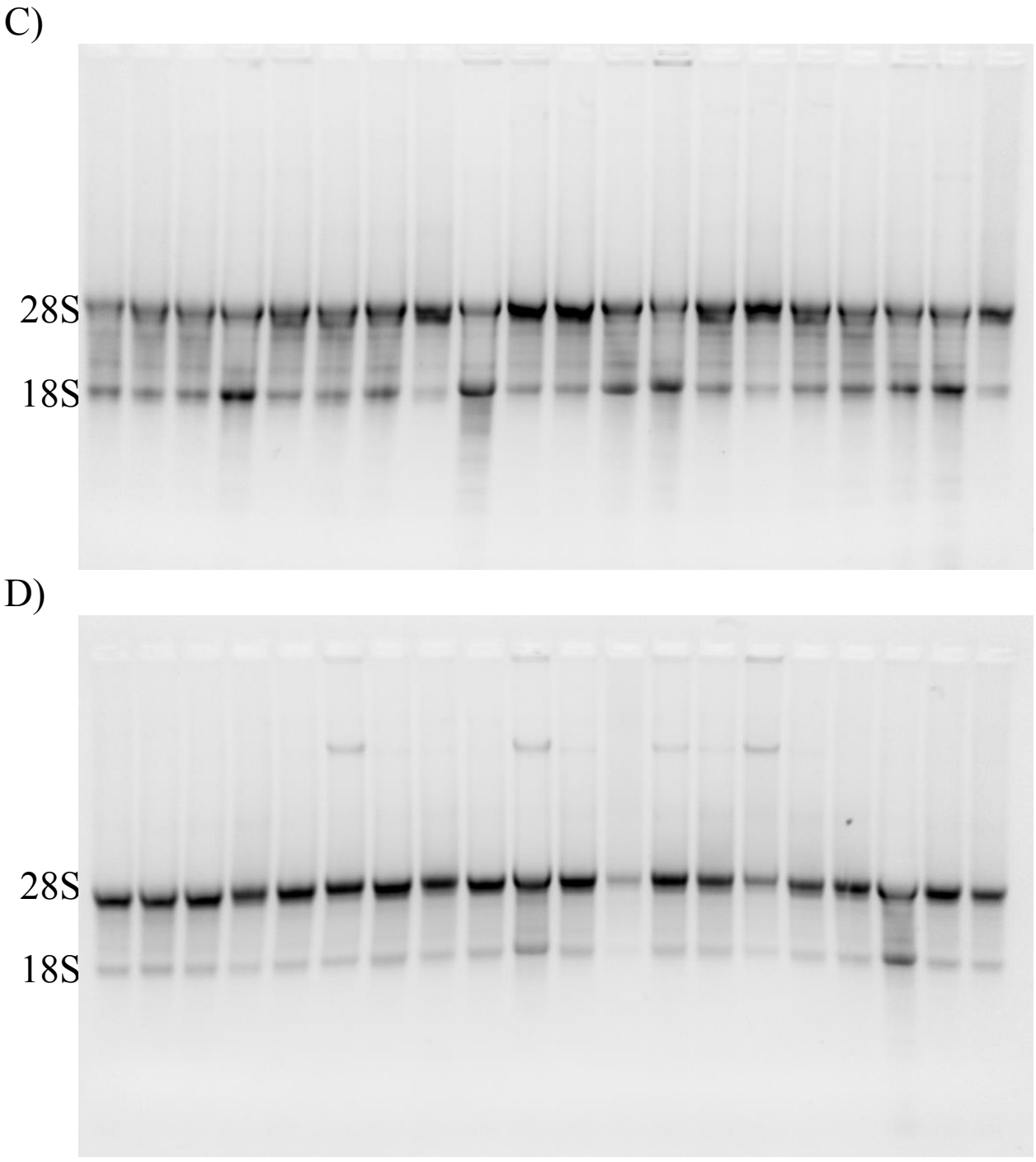


Figure 3.5. Images of agarose gels used to detect RNA integrity. In order to assess the integrity of extracted RNA from E19 and E22 placentas and fetal brains, an agarose gel (1.5%) was run. Ethidium bromide was incorporated into the gel for detection of DNA and RNA. The integrity of the RNA was assessed by evaluating 28S:18S ribosomal RNA ratio, where a 2:1 ratio was

interpreted as acceptable RNA integrity. The images depict E19 placentas (A), E19 brains (B), E22 placentas (C), and E22 brains (D). All bands depicting the 28S ribosomal RNA were much darker compared to the 18S ribosomal RNA, suggesting that the RNA was intact and the proportion of 28S:18S was appropriate for subsequent analyses of mRNA expression. Lanes that were empty were replaced (A, lane 9).

Table 3.3. Antibodies used for immunoblotting analyses on postnatal days 1, 7, and 21.

Immunoblotting Antibodies	Molecular Weight (kDa)	Concentration	Host	Catalogue Number	Company
Neuronal Growth					
Growth associated protein 43	43-53	1 µg/ml	Rabbit, polyclonal	AB16053	Abcam
Synaptogenesis					
Syntaxin	33	1 µg/ml	Rabbit, polyclonal	AB41453	Abcam
Synaptophysin	42	1/200	Rabbit, polyclonal	AB14692	Abcam
Oligodendroglial Cells and Maturation					
Olig2	32	1/2500	Rabbit, polyclonal	AB9610	Millipore
2',3'-Cyclic nucleotide 3'-phosphodiesterase	48	5 µg/ml	Mouse, monoclonal	AB6319	Abcam
Astrogliosis					
Glial fibrillary acidic protein	50	1/200	Rabbit, polyclonal	Z0334	DakoCytomation
BrSp					
Nuclear factor (erythroid-derived 2)-like 2	110	1 µg/ml	Mouse, monoclonal	MAB3925	R&D Systems
Loading Control					
β-actin	42	1/1000	Mouse, monoclonal	AB8226	Abcam

Table 3.3. Antibodies used for immunoblotting analyses on postnatal days 1, 7, and 21. Growth associated protein 43 was used for detected growth of neurons. Syntaxin and synaptophysin were both used to detect synaptogenesis. Olig2, a pan-oligodendroglial cell marker and 2',3'-cyclic nucleotide 3'-phosphodiesterase, a marker of differentiated oligodendrocytes, were used to analyze oligodendrocyte maturation. Glial fibrillary acidic protein was used to detect astrogliosis. Nuclear factor (erythroid-derived 2)-like 2 was used to analyze the effect of SFN on translocating the transcription factor the to the nucleus. β-actin was used as a loading control.

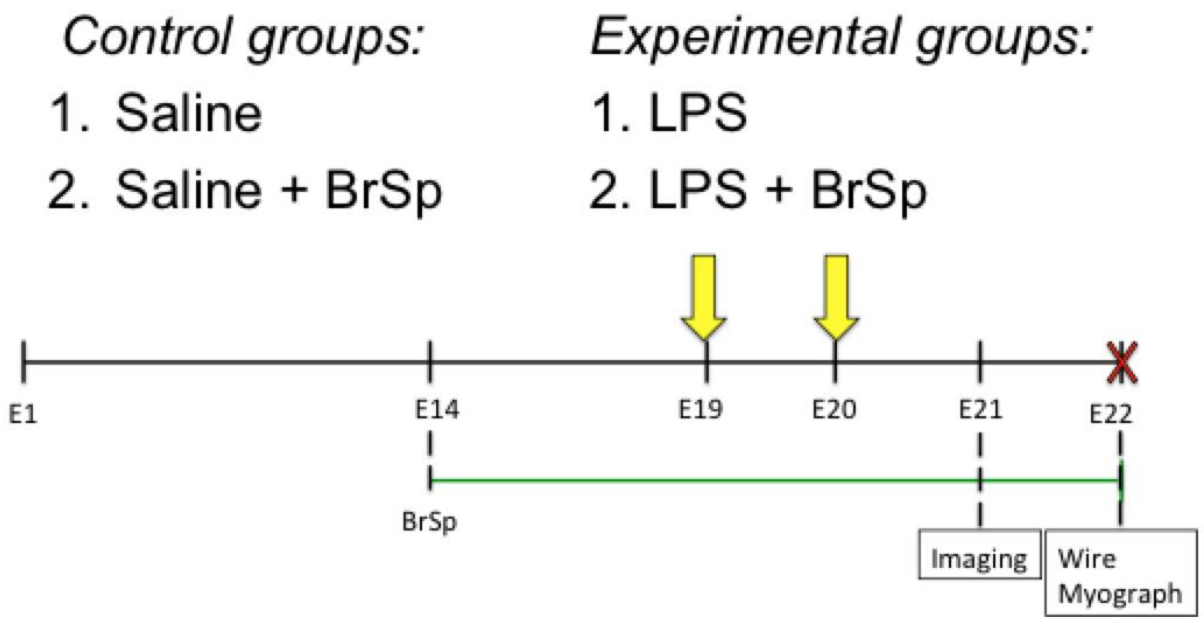


Figure 3.6. The methodological timeline for objective 3.The first day of pregnancy was defined as embryonic (E) day 1. On E14, dams were randomly selected to receive BrSp in addition to their diet. On E19 and E20, dams were randomly selected to receive either Saline (control) or LPS (treatment) i.p. injections every 12 hours over a period of two days. On E21, dams underwent Doppler imaging to analyze uterine and umbilical artery blood flow. On E22, dams were anesthetized and euthanized. Maternal blood, liver, kidneys, mesenteric arteries, aorta, and uterine arteries were collected and frozen (except for uterine arteries). Fetal placentas and brains were also collected and frozen for subsequent analyses.

4. RESULTS

4.1. OBJECTIVE 1

CP is the most common motor disorder in childhood. In clinical practice, developmental milestones are assessed, and those presenting with delayed or lack of acquisition are at 'high risk' for the diagnosis of subsequent neurodevelopmental disorders such as CP. [269,270] Moreover, by school age, children with CP present with cognitive difficulties in learning, memory, and executive functioning. [271,272] The pathological correlate associated with CP is white matter injury, also known as periventricular leukomalacia. White matter injury arises from *in utero* insult during the period from 24-32 weeks gestation. [36,37,42,43] During this timeframe, pre-oligodendrocytes are the predominant form of cells surrounding the ventricular region. [36,37,42,43] These cells do not have a mature antioxidant system and are vulnerable to exogenous insults. [36,37,42,43] Injury to these cells results in death and/or a maturational blockade, ultimately leading to reduced myelination in the developing brain. [36,37,42,43] At present, no preventive treatment is available. Thus, the objective of this study was to replicate a model of CP induced by *in utero* inflammation, and investigate the neuroprotective effects of BrSp.

4.1.1. Maternal Body Weight and Temperature Responses

Maternal temperature (Figure 4.1A) was recorded prior to every injection to monitor the physiologic response to both LPS and BrSp. Baseline body temperature was recorded prior to the first injection. Rats in the LPS and LPS + BrSp initially displayed a hypothermic response at 6 and 12 hours after the first LPS injection. There was a significant main effect of treatment at 12

hours ($F(1,18)=4.9$, p=0.04). LPS (-0.4 ± 0.1°C) and LPS + BrSp (-0.5 ± 0.3°C) dams' body temperature was lower whereas Saline (0.01 ± 0.3°C) and Saline + BrSp (0.1±0.1°C) dams' body temperature remained close to baseline recordings. Between 12 and 24 hours, LPS + BrSp dams body temperature recovered to baseline levels however, LPS dams were still hypothermic. A significant main effect of treatment at 24 hours was detected ($F(1,17)=4.5$, p=0.05). LPS (-0.6 ± 0.3°C) remained lower whereas LPS + BrSp (-0.1 ± 0.1°C), Saline (0.1 ± 0.2°C), and Saline + BrSp (0.04 ± 0.1°C) body temperature were similar to baseline temperatures. By 48 hours, all body temperatures were unchanged from their respective baseline recordings.

Maternal weights (Figure 4.1B) were recorded prior to every injection to monitor changes in body weight following LPS and BrSp exposure. The initial body weight was recorded prior to the first injection and used as a baseline. Changes to body weight after the baseline recording was calculated as a percent weight change to reveal differences due to the variability of starting weight of the dams as a result of litter size and weights. At 12 hours after the first injection, no significant changes to weight were observed in any of the groups of rats. At 24 hours, Saline and Saline + BrSp dams gained weight. LPS dams showed a reduction in body weight whereas LPS + BrSp dams did not exhibit a change in body weight compared to the baseline. There was a significant main effect of treatment ($F(1,21)=25.3$, p=0.0001). LPS (-1.5 ± 0.8%) dams had a significant change in weight compared to Saline dams (2.9 ± 0.7%, Tukey's p<0.001). At 36 hours, Saline and Saline + BrSp dams continued to gain weight, whereas LPS and LPS + BrSp weights were not different from baseline. There was a significant main effect of treatment at 36 hours ($F(1,21)=6.7$, p=0.02) however, no group differences were detected.

4.1.2. Litter Size and Birth Weights

Following birth, the number of viable pups (Figure 4.2A) was counted to determine whether exposure to either LPS or BrSp affected fetal mortality. There was a significant main effect of treatment ($F(1,25)=5.2$, $p=0.03$). LPS (10.3 ± 1.5 pups) and LPS + BrSp (11.0 ± 1.2 pups) dams had smaller litter sizes compared to Saline (13.3 ± 1.0 pups) and Saline + BrSp (13.1 ± 0.8 pups) dams.

The birth weights of pups were recorded (Figure 4.2B). There was a significant main effect of treatment ($F(1,23)=18.5$, 0.0003), Diet ($F(1,23)=6.5$, $p=0.02$), and an interaction effect ($F(1,23)=7.4$, $p=0.01$). LPS (5.1 ± 0.2 g) pups were significantly smaller than Saline (6.3 ± 0.2g, Tukey's $p<0.001$), Saline + BrSp (6.2 ± 0.2g, Tukey's $p<0.001$) and LPS + BrSp (6.0 ± 0.1 g, $p<0.01$). The birth weights of LPS + BrSp pups were not different from Saline and Saline + BrSp pups.

4.1.3. Postnatal Growth

The body weights of the pups were recorded on PD7 (Figure 4.2C) and PD21 (Figure 4.2D) to determine changes to body weights. On PD7, there was a significant main effect of treatment ($F(1,23)=15.9$, $p=0.0006$). LPS (11.4 ± 1.1g) and LPS + BrSp (13.6 ± 0.3g) weighed less than Saline (15.0 ± 0.5g) and Saline + BrSp (15.5 ± 0.4g) pups. Post-hoc group analyses revealed that LPS pups weighed significantly less than Saline (Tukey's $p<0.01$) and Saline + BrSp (Tukey's $p<0.01$). Although LPS weights did not differ from LPS + BrSp pups, LPS +

BrSp pups were not different from Saline and Saline + BrSp pups. On PD21, the pups from all four groups were of similar weight indicating that by three weeks of age, the LPS pups displayed 'catch up growth'.

4.1.4. Developmental Reflexes

Developmental reflexes were examined to identify whether this inflammatory model was able to produce delays or lack of acquisition of specific developmental milestones. Forelimb grasp was examined on PD3 and all pups, irrespective of group, were able to perform this task. Similar to forelimb grasping, no differences among groups in the hindlimb grasp reflex were detected. However, upon analysis of hindlimb placing (Figure 4.3A), a significant main effect of treatment ($F(1,23)=6.8$, $p=0.02$) was detected. LPS (5.1 ± 0.3 days) and LPS + BrSp (4.5 ± 0.2 days) were delayed in acquiring this reflex compared to Saline (4.3 ± 0.1 days) and Saline + BrSp (4.3 ± 0.0 days). LPS pups were significantly delayed in acquiring hindlimb placing compared Saline (Tukey's $p<0.05$) and Saline + BrSp (Tukey's $p<0.05$) pups. Although LPS and LPS + BrSp pups were not different from each other, LPS + BrSp pups were not different from Saline and Saline + BrSp. Cliff avoidance testing (Figure 4.3B) also showed a significant main effect of treatment ($F(1,25)=6.0$, $p=0.02$). LPS (5.8 ± 0.4 days) pups were significantly delayed in acquiring this reflex compared to Saline (4.4 ± 0.2 days, Tukey's $p<0.05$). Although no differences were found between LPS and LPS + BrSp, LPS + BrSp pups were not different from Saline and Saline + BrSp. Analysis of gait (Figure 4.3C) revealed a significant effect of treatment ($F(1,24)=15.1$, $p=0.0007$), diet ($F(1,24)=6.3$, $p=0.02$), and an interaction between treatment and diet ($F(1,24)=9.5$, $p=0.005$). LPS pups (9.7 ± 0.4 days) were significantly delayed

in attaining this reflex compared to Saline (7.5 ± 0.3 days, Tukey's $p<0.001$), Saline + BrSp (7.7 ± 0.3 days, Tukey's $p<0.001$), and LPS + BrSp (7.9 ± 0.2 days, Tukey's $p<0.01$). Following analysis of righting (Figure 4.3D), a sex effect was detected ($F(1,43)=16.3$, p-0.0002), thus, males and females were analyzed separately. No differences were observed in males. In females, a main effect of Diet ($F(1,21)=11.8$, $p=0.002$) and an interaction effect ($F(1,21)=15.6$, $p=0.0007$) were detected. LPS + BrSp pups (4.9 ± 0.4 days) acquired this reflex earlier than LPS pups (6.7 ± 0.6 days, Tukey's $p<0.05$). Accelerated righting testing revealed a significant main effect of treatment ($F(1,25)=4.51$, $p=0.04$). LPS (17.4 ± 0.6 days) and LPS + BrSp (16.9 ± 0.2 days) performed this task later than Saline (16.0 ± 0.4 days) and Saline + BrSp (16.4 ± 0.5 days) pups.

4.1.5. Maturation

The auditory startle response was not affected by treatment or diet. There was a significant interaction effect of treatment and diet ($F(1,24)=5.8$, $p=0.02$) on posture (Figure 4.3E). LPS + BrSp (14.9 ± 0.4 days) pups attained a mature posture earlier than LPS (17.0 ± 0.4 days, Tukey's $p<0.05$). There was a significant main effect of diet ($F(1,23)=4.71$, $p=0.04$) on eye opening. Saline + BrSp (15.9±0.1 days) and LPS + BrSp (15.4±0.2 days) pups acquired this maturation sign earlier than Saline (16.0±0.2 days) and LPS (16.0±0.1 days).

4.1.6. Open Field Analyses

Five parameters were testing in the open field analyses: rearing, head lifts, urination and defecation, grooming, and ambulation. No differences were observed among the groups in

rearing (Figure 4.4A), head lifts, nor the amount of defecation and urination. There was a significant main effect of treatment ($F(1,24)=5.9$, $p=0.02$) and diet ($F(1,24)=9.9$, $p=0.004$) on grooming (Figure 4.4B) behaviour. LPS (2.2 ± 0.4 times) groomed significantly more times compared to Saline (1.3 ± 0.1 times, Tukey's $p<0.05$), Saline + BrSp (1.0 ± 0.1 times, Tukey's $p<0.01$), and LPS + BrSp (1.1 ± 0.1 times, Tukey's $p<0.01$). Assessment of ambulation, (Figure 4.4C) defined as the number of times the pups crossed a square, revealed a significant main effect of treatment ($F(1,25)=4.9$, $p=0.04$). LPS (73.0 ± 5.8 squares) and LPS + BrSp (76.2 ± 6.5 squares) crossed fewer squares than Saline (90.3 ± 4.7 squares) and Saline + BrSp (92.9 ± 12.3 squares). The amount of time (seconds) spent in the center of the open field box (Figure 4.4D) was also assessed. A significant interaction was found ($F(1, 23)=4.9$, $p=0.04$). LPS (16.4 ± 2.1 seconds) pups spent less time in the center compared to Saline (25.4 ± 2.2 seconds), Saline + BrSp (23.0 ± 3.6 seconds), and LPS + BrSp (25.7 ± 2.3 seconds) pups, however, post-hoc analyses did not reveal group differences.

4.1.7. Pathological Assessment

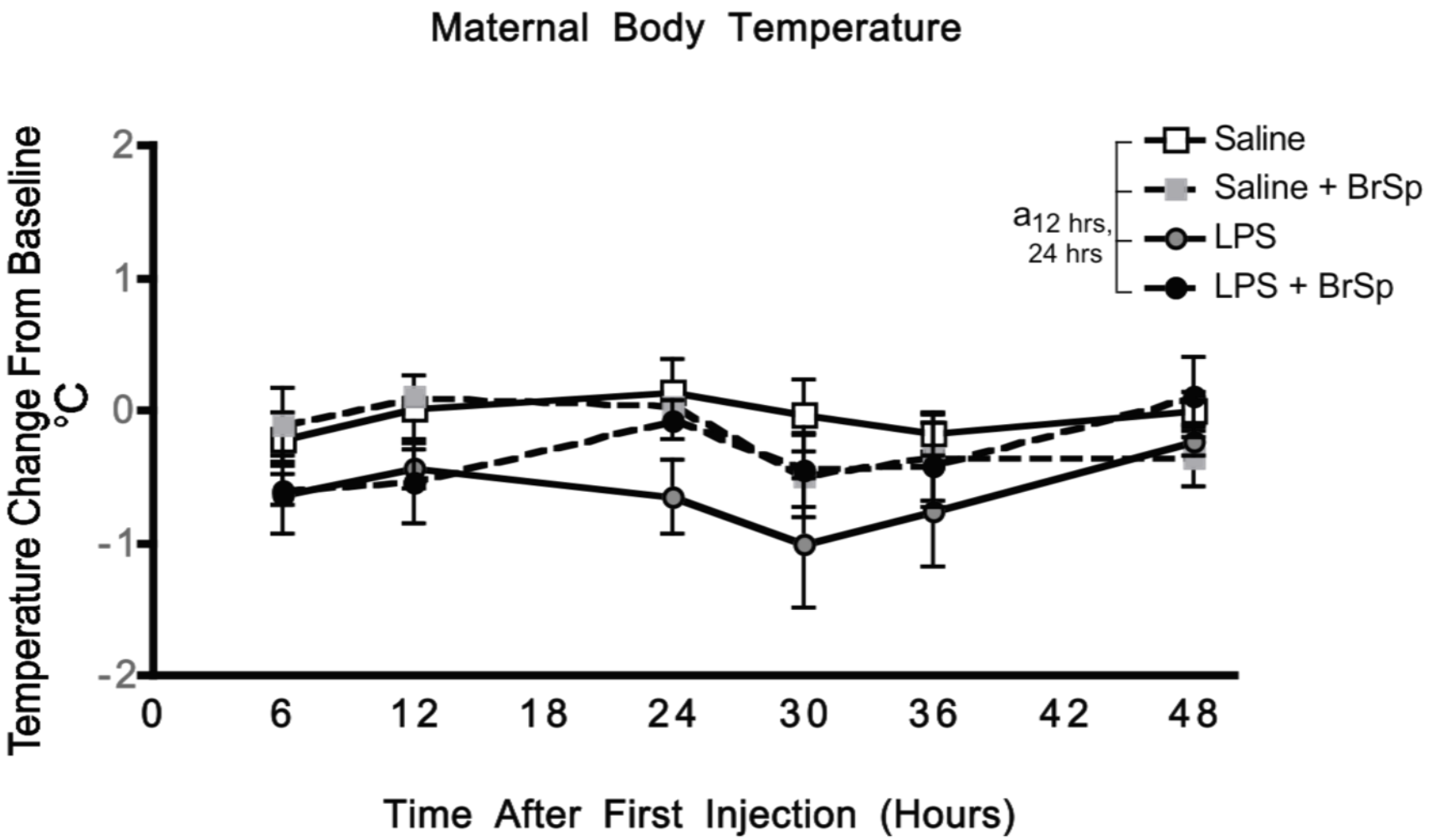
The first pathological analyses were evaluating changes in brain and lateral ventricular volume. In clinical settings, white matter injury often leads to ventriculomegaly due to the loss of periventricular parenchyma and thus, reduction in brain volume and enlargement of ventricles. No significant effects of treatment or diet on brain volume or lateral ventricular volume were identified. Fractin staining was used as a marker of apoptotic axons, or gray matter injury; no differences were identified (data not shown).

White matter injury is the hallmark feature of CP, and thus, the next assessment was the evaluation of oligodendrocytes and myelination. Olig2, a pan-oligodendroglial stain (Figure 4.5A) was used to count the number of oligodendrocytes present on two main white matter tracts, the corpus callosum (Figure 4.5B) and the cingulum (Figure 4.5C). Upon counting, no differences were observed. Immunodensity of myelin basic protein (Figure 4.6A), the predominant protein found in myelin, was measured and a significant main effect of treatment ($F(1,24)=5.7$, p=(0.03) was observed. LPS (0.4 ± 0.04 absorbance units, a.u.) and LPS + BrSp (0.5 ± 0.1 a.u.) samples exhibited a reduced myelin basic protein immunodensity compared to Saline (0.6 ± 0.03 a.u.) and Saline + BrSp (0.5 ± 0.1 a.u.) in the corpus callosum (Figure 4.6B). However, Tukey's test did not detect group differences. Similarly, no differences were detected following myelin basic protein analyses in the cingulum (Figure 4.6C).

4.1.8. Summary

This model of fetal inflammation reproduced developmental delay and behavioural abnormalities similar to those observed in the human population with CP. Some of these developmental delays were attenuated following BrSp dietary supplementation of the pregnant and lactating dam. Furthermore, a reduction in myelination was observed, without any changes in the number of oligodendrocytes present, supporting the notion that fetal inflammation causes oligodendrocyte maturation blockade. This is also in agreement with the literature showing white matter injury as a result of maturation blockade. BrSp did not have an effect on the myelination, suggesting that the neuroprotection afforded by BrSp may be mediated by a different mechanism.

A)


Maternal Body Temperature
Temperature Change From Baseline °C
2
1
0
-1
-2
0
6
12
18
24
30
36
42
48
Time After First Injection (Hours)
Saline
Saline + BrSp
LPS
LPS + BrSp
a 12 hrs, 24 hrs

B)

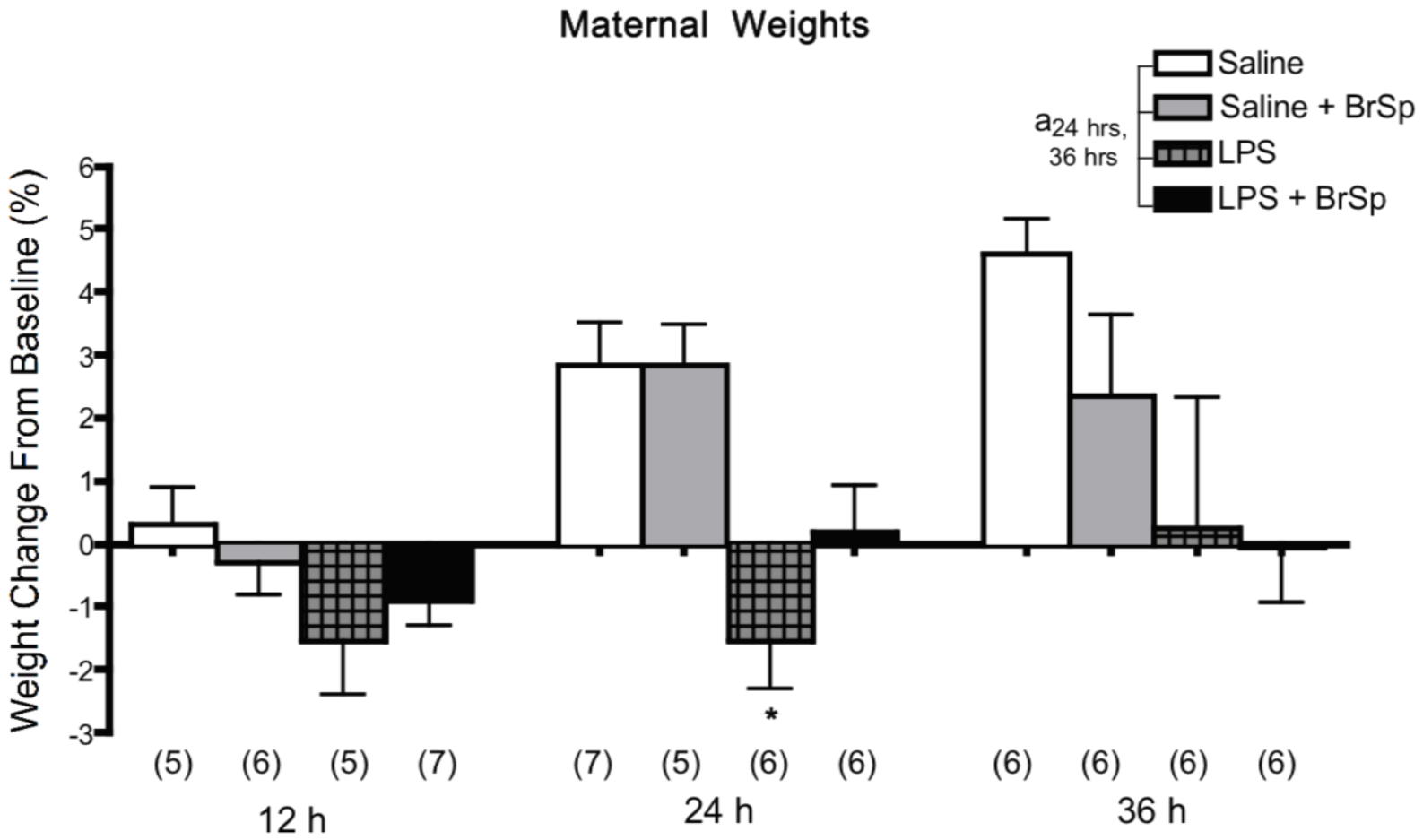
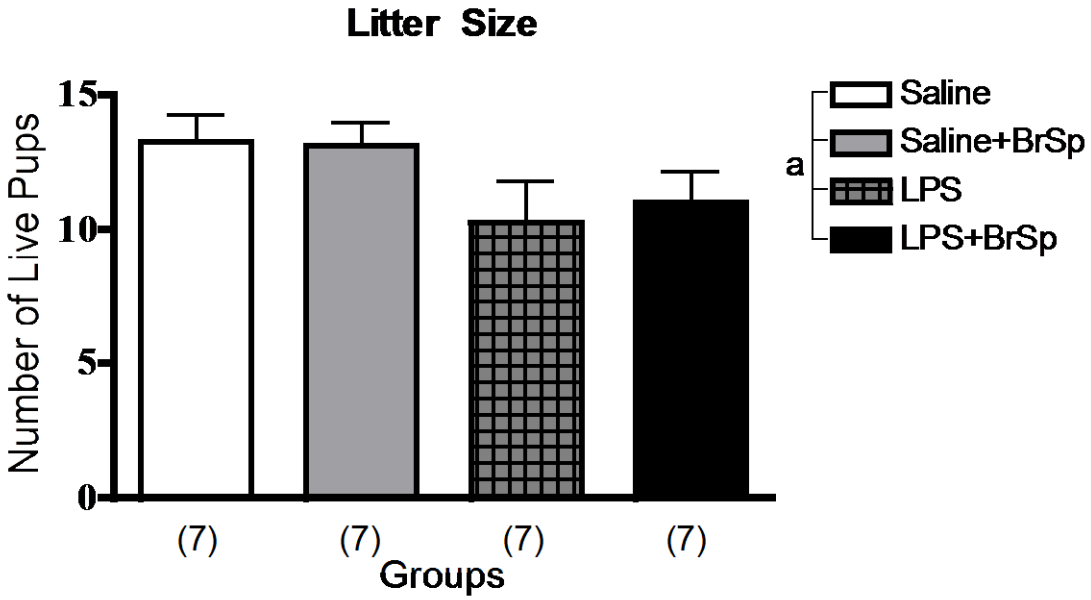
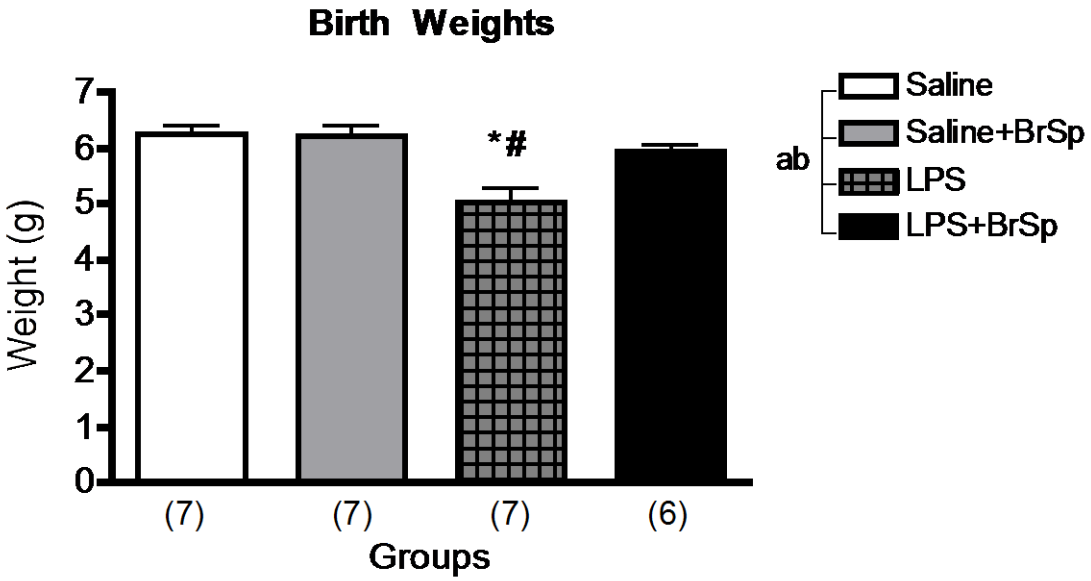

Maternal Weights
Saline
Saline + BrSp
LPS
LPS + BrSp
a 24 hrs, 36 hrs
Weight Change From Baseline (%)
6
5
4
3
2
1
0
-1
-2
-3
*
(5) (6) (5) (7) (7) (5) (6) (6) (6) (6) (6) (6)
12 h
24 h
36 h

Figure 4.1. Body temperature and weights of pregnant rats over the duration of Saline or LPS intraperitoneal injections. Maternal temperature (A) and percent change in body weight (B) were compared to baseline. For the maternal temperature analyses (A), there was a significant main effect of Treatment at 12 and 24 hours. For maternal percent body weight change (B), there was a significant main effect of Treatment at 24 and 36 hours. Data are presented as mean ± SEM. For the maternal temperature analyses, *n*=4-6 pregnant rats were used. For maternal weights, numbers in brackets on the x-axis represent *n* numbers. a = significant main effect of Treatment. * = significant difference between LPS and Saline dams.

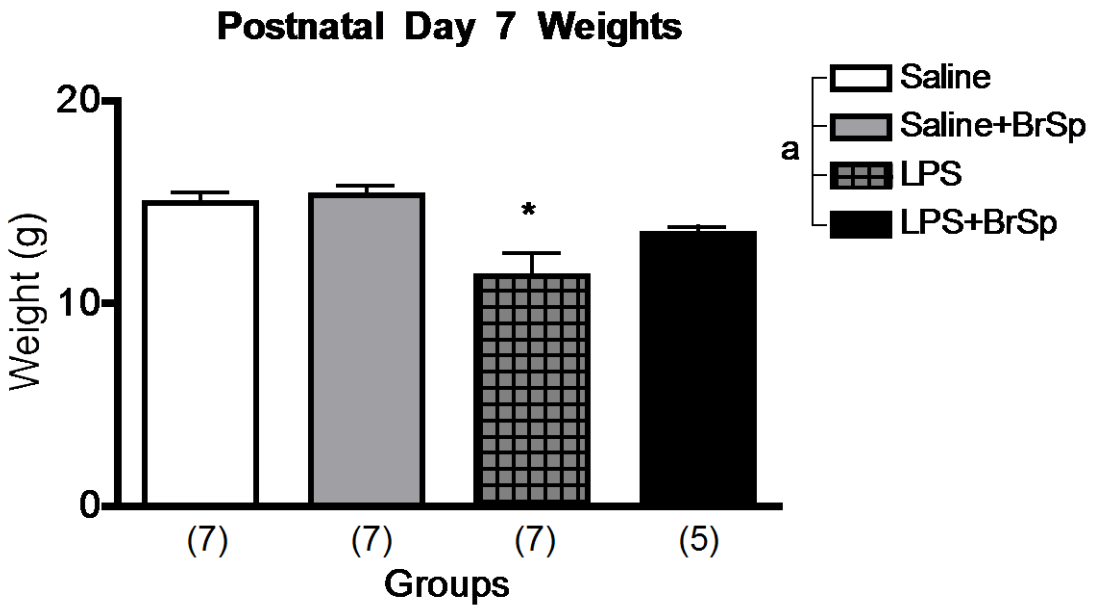
A)


Litter Size
Number of Live Pups
15
10
5
0
(7)
(7)
(7)
(7)
Groups
a
Saline
Saline+BrSp
LPS
LPS+BrSp

B)


Birth Weights
Weight (g)
7
6
5
4
3
2
1
0
*#
(7)
(7)
(7)
(6)
Groups
ab
Saline
Saline+BrSp
LPS
LPS+BrSp

C)



D)

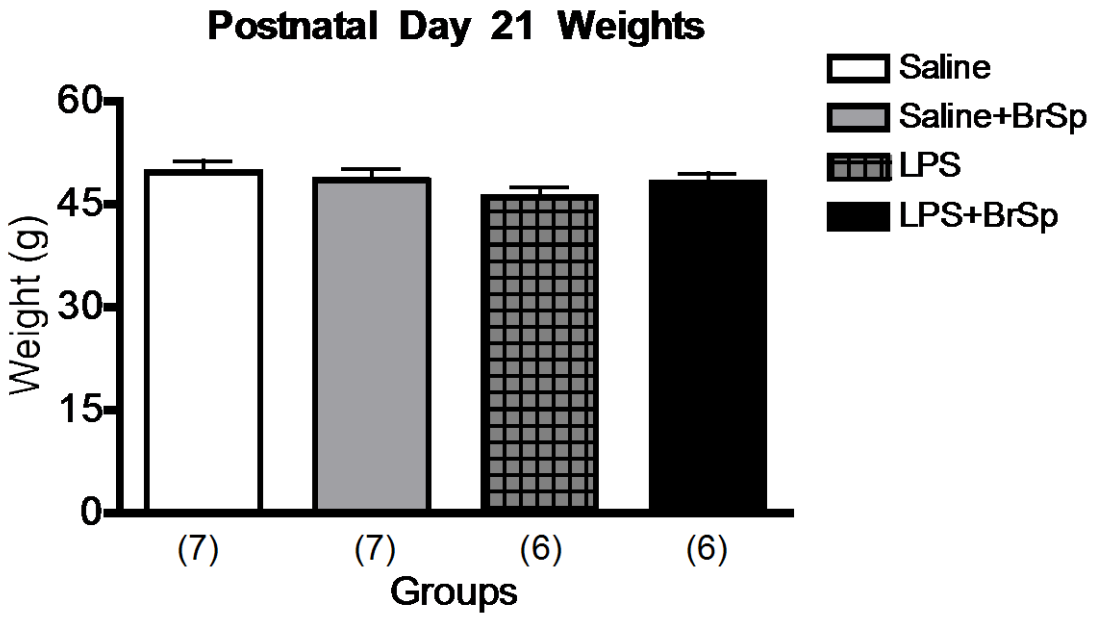
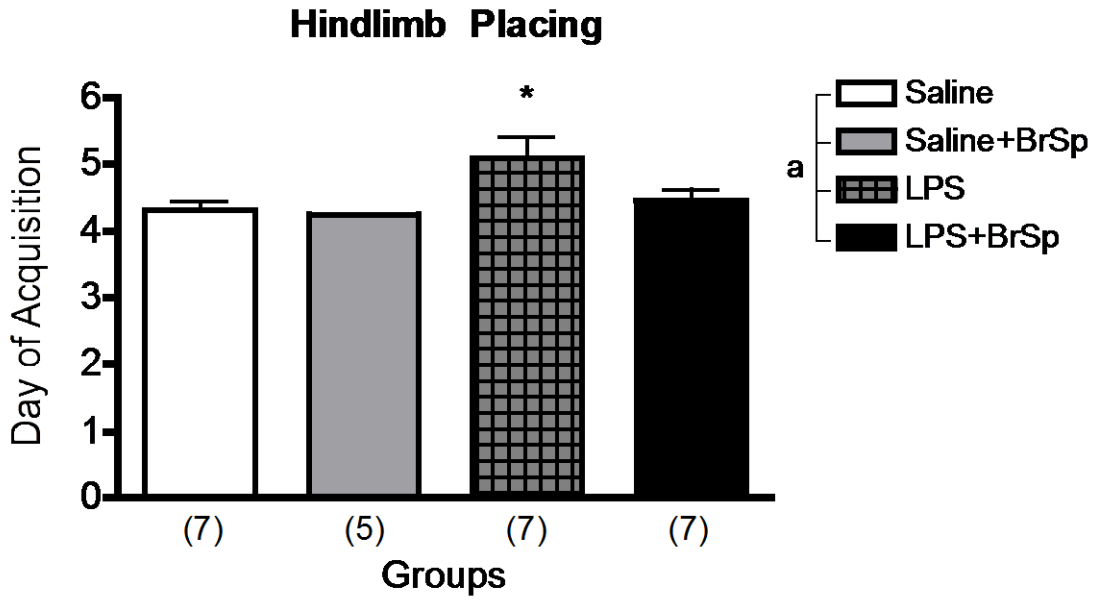


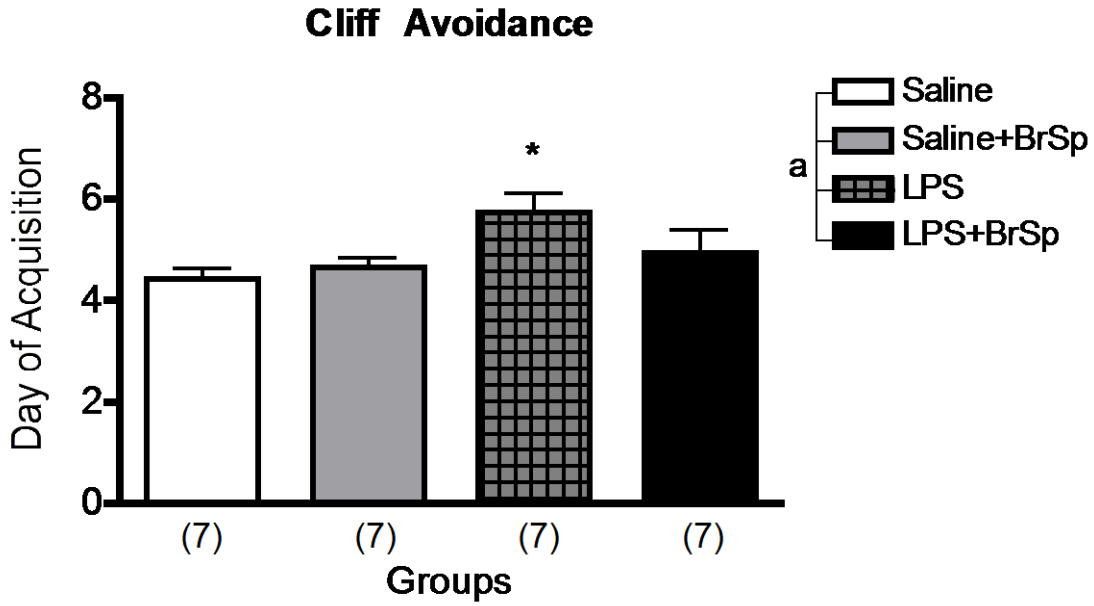
Figure 4.2. Litter size and weights of the offspring on postnatal day 1, 7, and 21 over the duration of the experiments. The numbers of viable pups were counted to determine the litter size (A) of each group. There was a significant main effect of Treatment on litter size (A). LPS and LPS + BrSp dams had smaller litters compared to Saline and Saline + BrSp dams. LPS offspring had

significantly smaller birth weights (B) compared to Saline, Saline + BrSp, and LPS + BrSp pups. On PD7, the weights (C) of the LPS pups were still significantly lower compared to Saline pups. By PD21 (D), LPS pups had achieved ‘catch-up growth’. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent *n* numbers. a = significant main effect of Treatment. b = significant main effect of Diet. * = significant difference between LPS and Saline pups. # = significant difference between LPS and LPS + BrSp pups.

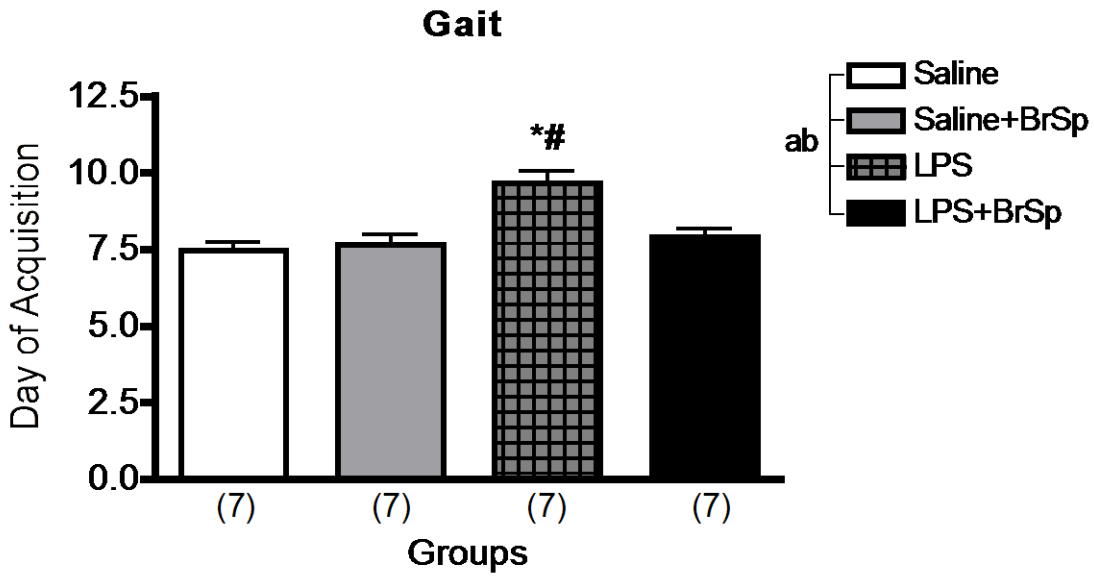
A)


Hindlimb Placing
Day of Acquisition
0
1
2
3
4
5
6
*
(7)
(5)
(7)
(7)
Groups
a
Saline
Saline+BrSp
LPS
LPS+BrSp

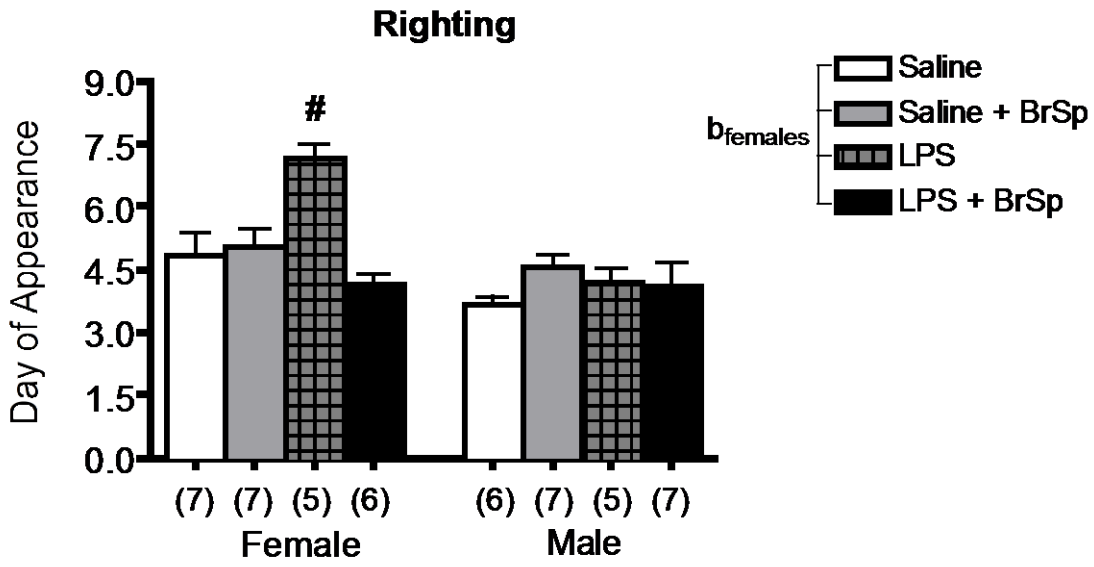
B)


Cliff Avoidance
Day of Acquisition
0
2
4
6
8
*
(7)
(7)
(7)
(7)
Groups
a
Saline
Saline+BrSp
LPS
LPS+BrSp

C)


Gait
Day of Acquisition
12.5
10.0
7.5
5.0
2.5
0.0
*#
(7)
(7)
(7)
(7)
Groups
ab
Saline
Saline+BrSp
LPS
LPS+BrSp

D)


Righting
Day of Appearance
9.0
7.5
6.0
4.5
3.0
1.5
0.0
#
(7) (7) (5) (6)
Female
(6) (7) (5) (7)
Male
b females
Saline
Saline + BrSp
LPS
LPS + BrSp

E)

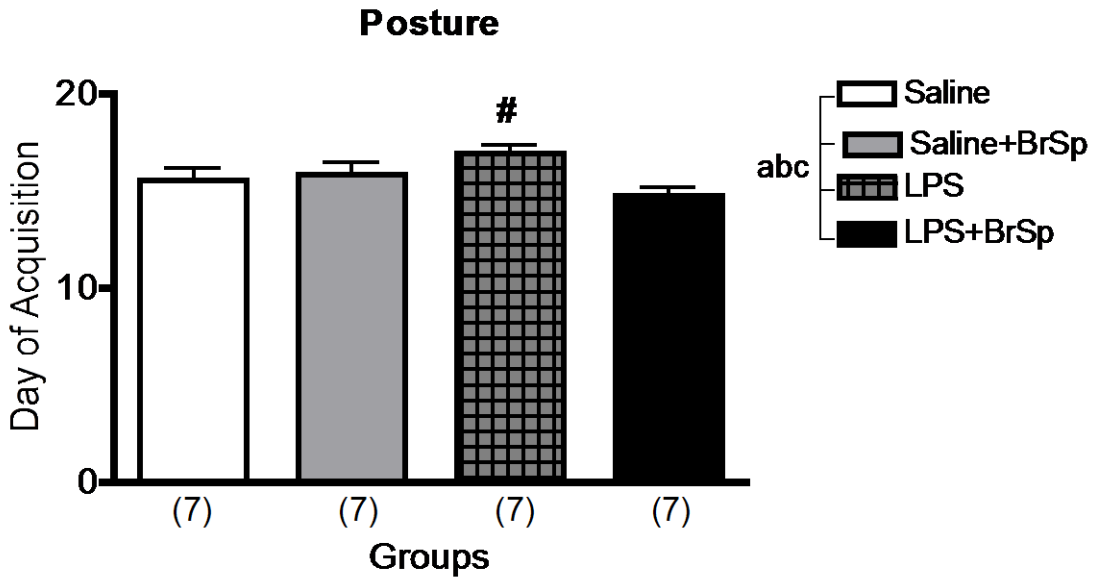
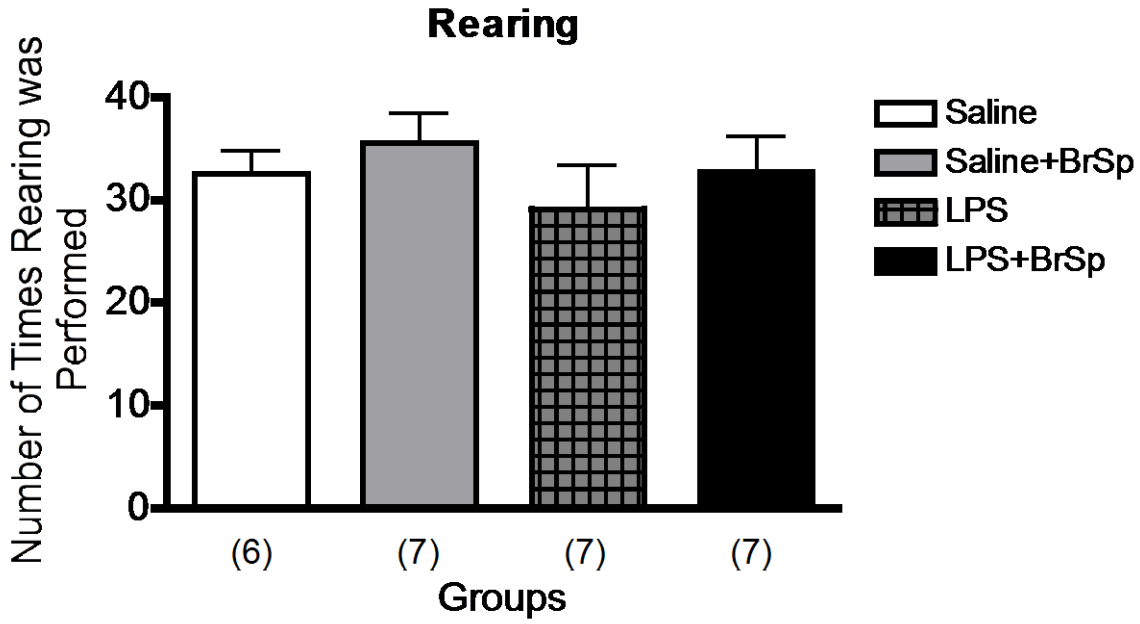


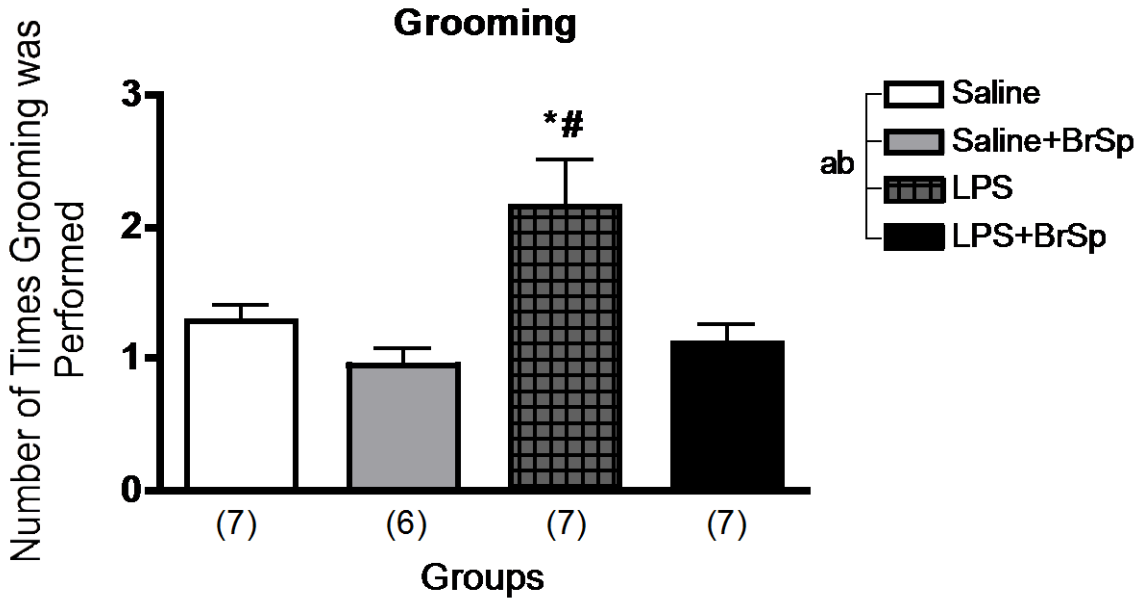
Figure 4.3. Neurodevelopment reflexes and posture maturation outcomes in the offspring. Hindlimb placing (A) was assessed and a significant main effect of Treatment was detected. LPS pups were significantly delayed in acquiring this reflex compared to Saline pups. A significant main effect of Treatment was detected for cliff avoidance (B). LPS pups were significantly delayed in acquiring this reflex compared to Saline. Gait (C) analysis also revealed a significant main effect of both Treatment and Diet. LPS pups were significantly delayed in acquiring this reflex compared to both Saline and LPS + BrSp pups. A sex effect was detected for righting (D) therefore, males and females were analyzed separately. In the females, a main effect of Diet was detected. Females in the LPS group were significantly delayed in acquiring this reflex compared to LPS + BrSp female pups. No effects were detected in the males. Posture (E) was evaluated to investigate the maturation of the pups. A significant main effect of Treatment, Diet and an interaction effect were identified. LPS + BrSp pups acquired this maturation sign earlier than LPS pups. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. b = significant main

effect of diet. c = significant interaction effect. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp). # = significant difference between LPS and LPS + BrSp.

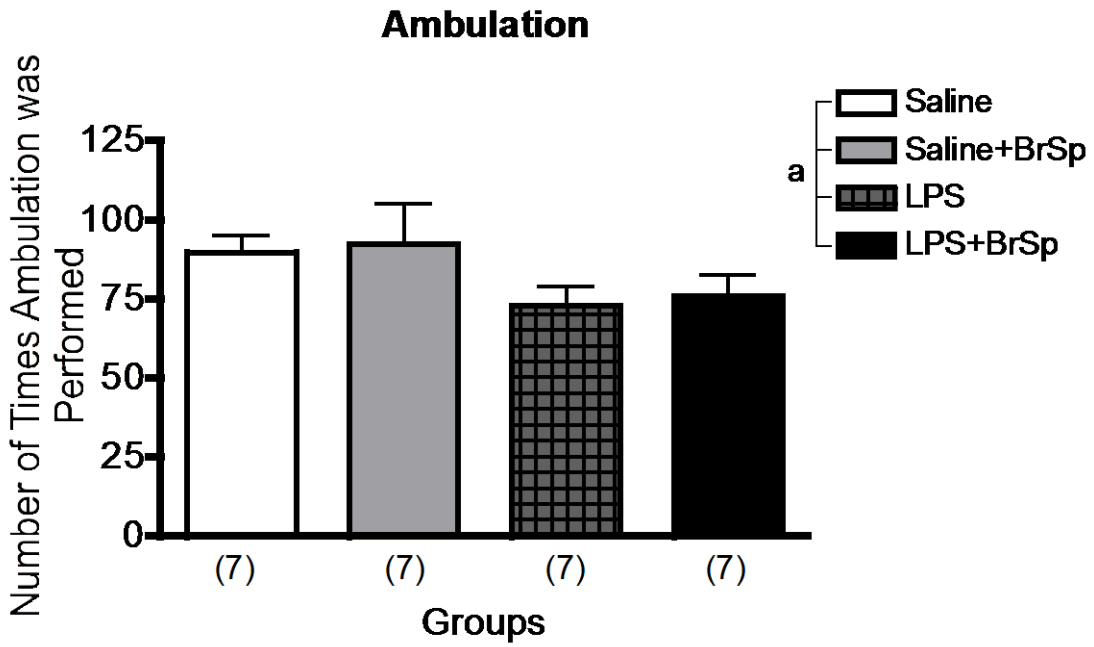
A)


Rearing
Number of Times Rearing was Performed
40
30
20
10
0
(6)
(7)
(7)
(7)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

B)


Grooming
Number of Times Grooming was Performed
3
2
1
0
*#
(7)
(6)
(7)
(7)
Groups
ab
Saline
Saline+BrSp
LPS
LPS+BrSp

C)



D)

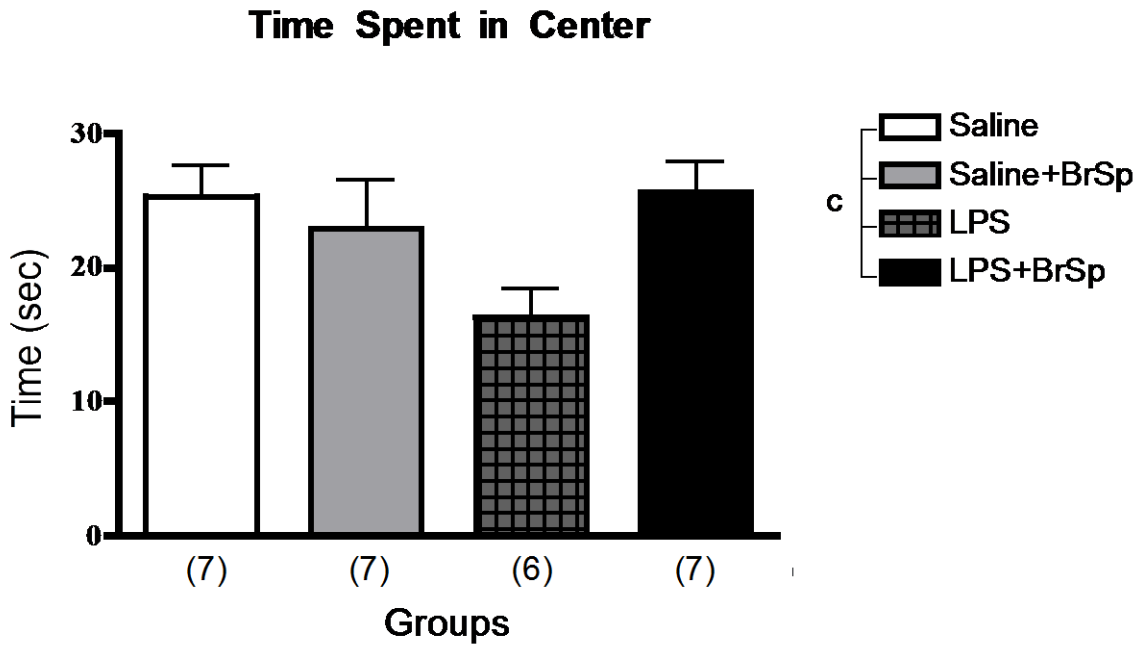
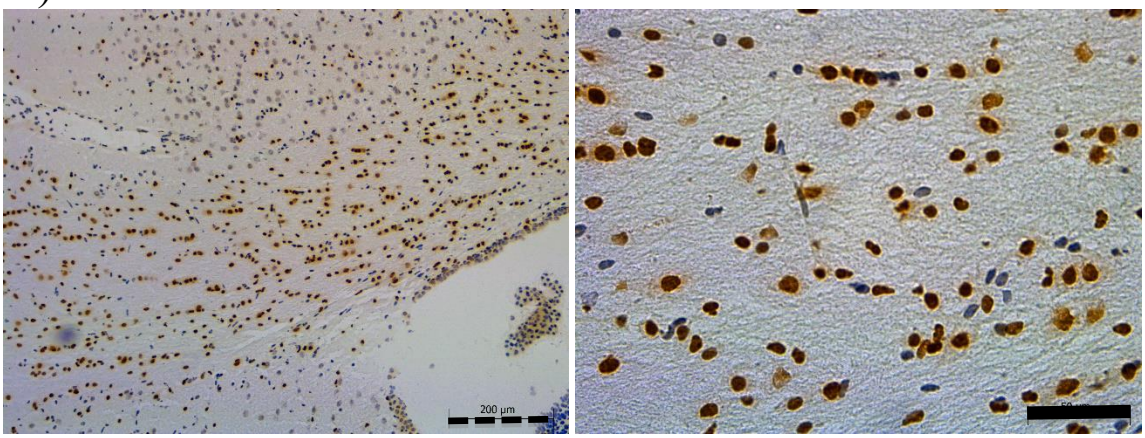


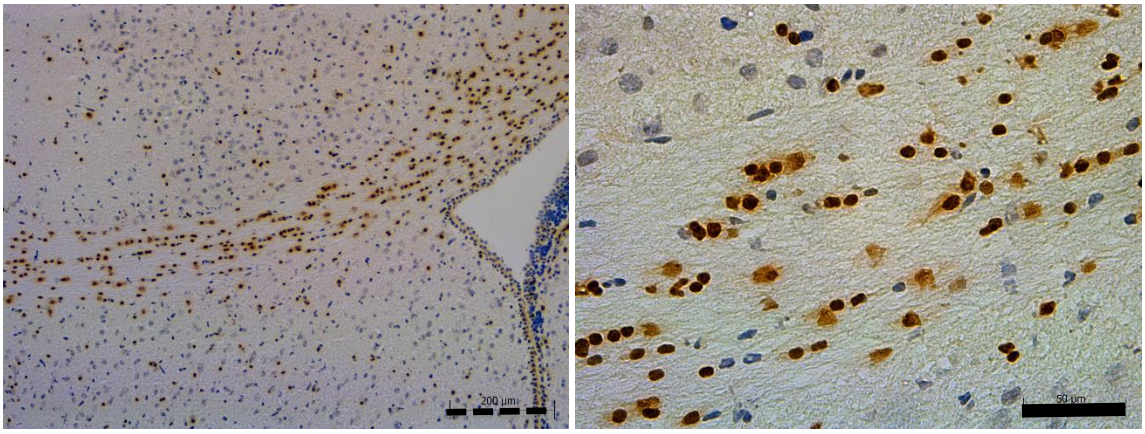
Figure 4.4. Open field behaviour analyses of anxiety and exploratory behaviour in the pups. No differences were detected in rearing (A). Grooming (B) analysis revealed a significant main effect of Treatment and Diet. LPS pups groomed significantly more times than Saline and LPS +

BrSp pups. LPS and LPS + BrSp pups ambulated significantly less than Saline and Saline + BrSp pups (C). No differences were observed among groups in the time spent in the center of the open field box (D). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. b = significant main effect of diet. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp). # = significant difference between LPS and LPS + BrSp.

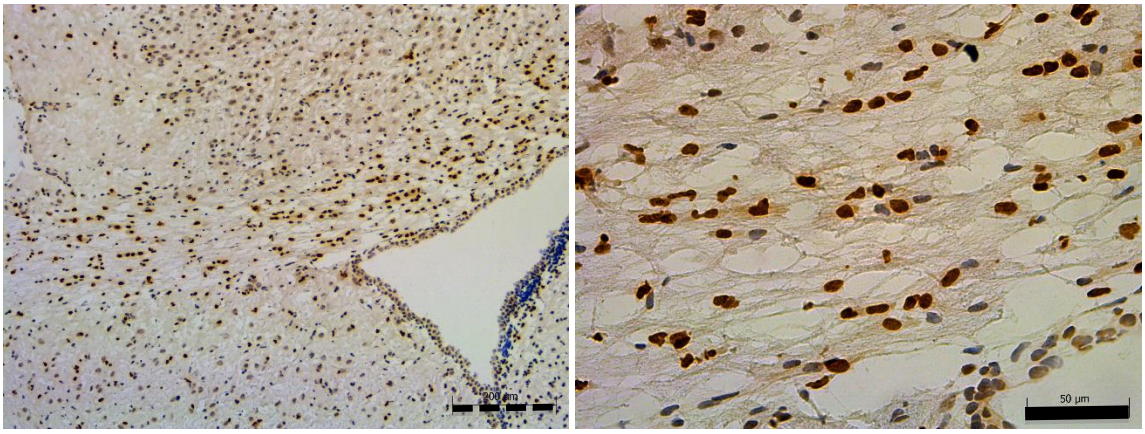
A)



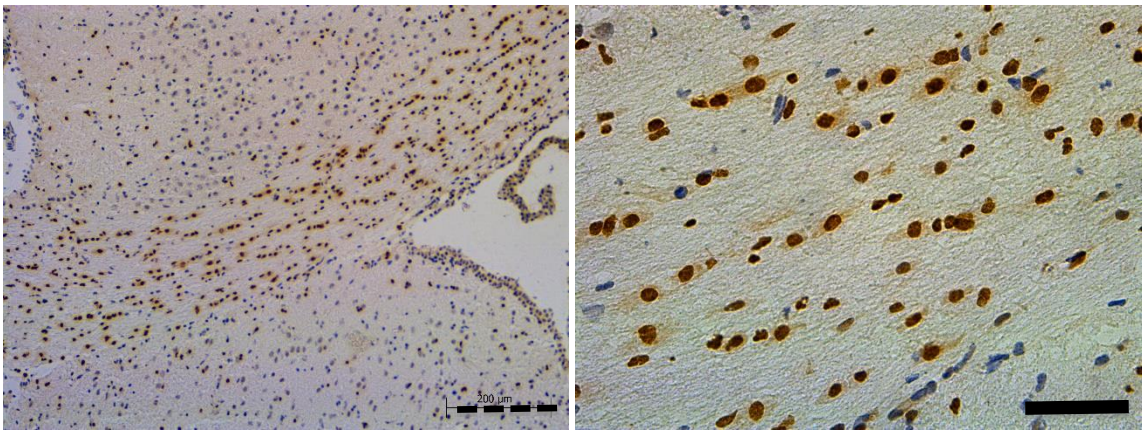
Saline



Saline + BrSp

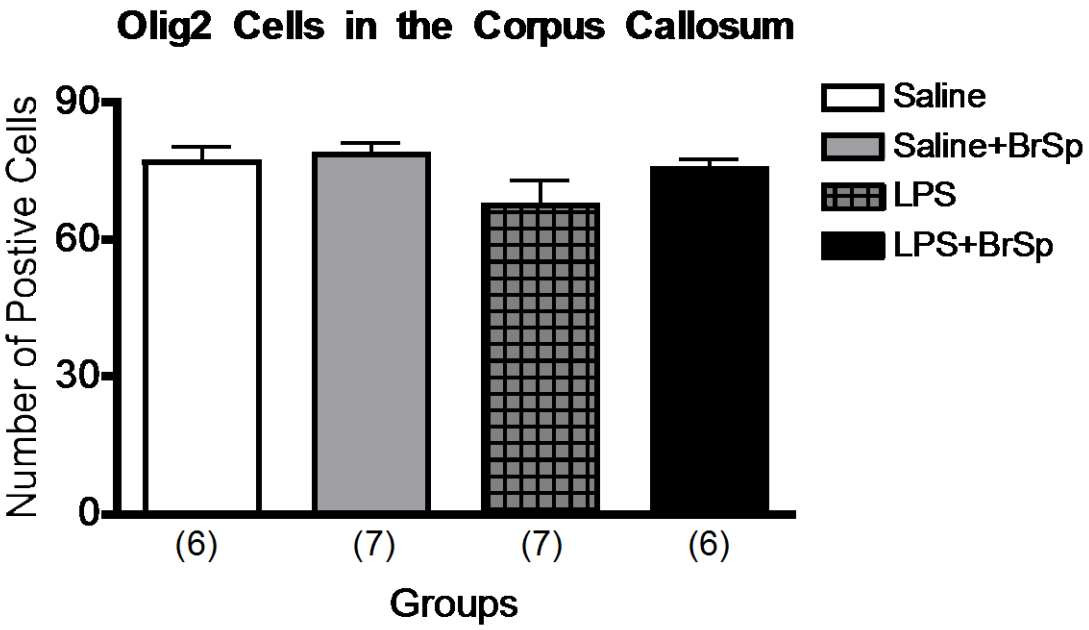


LPS


200 µm

LPS + BrSp

B)


Olig2 Cells in the Corpus Callosum
Number of Postive Cells
90
60
30
0
(6)
(7)
(7)
(6)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

C)

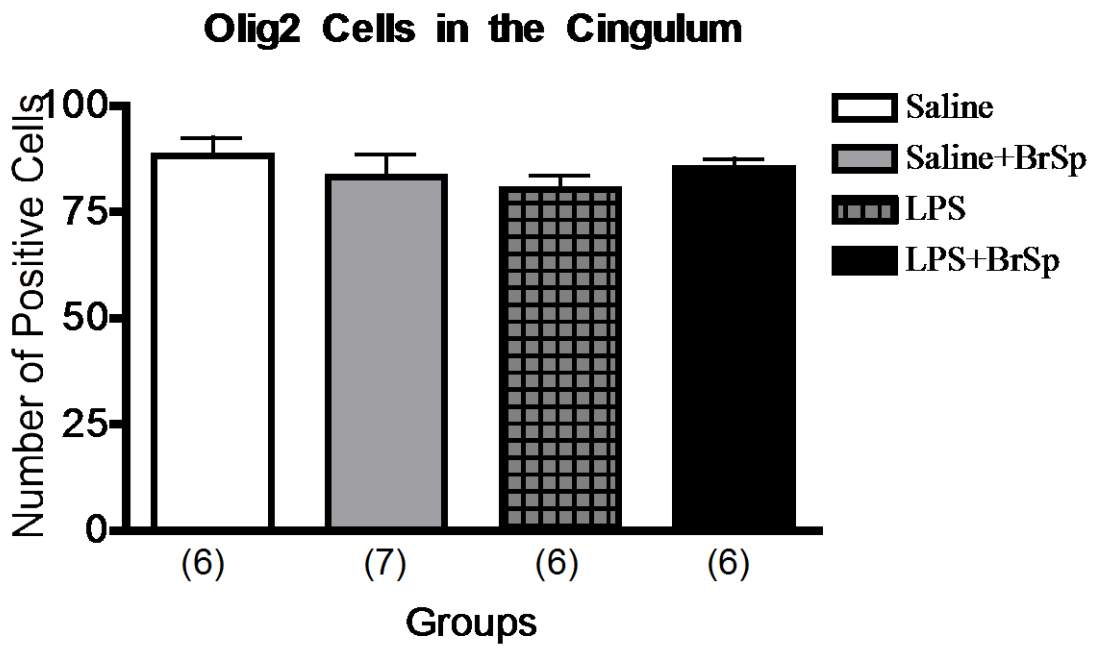
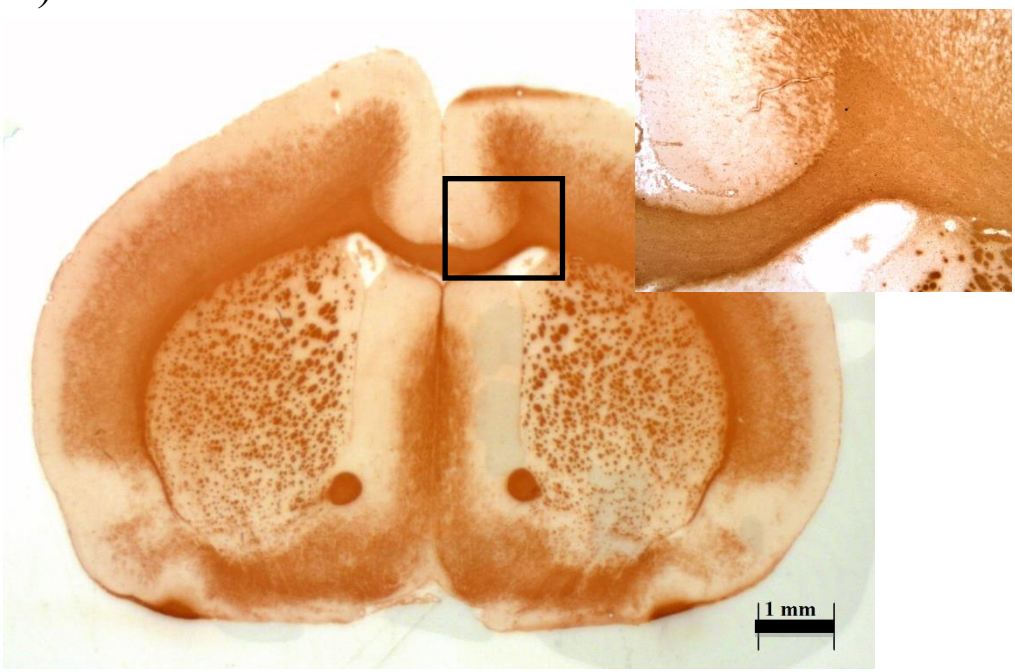
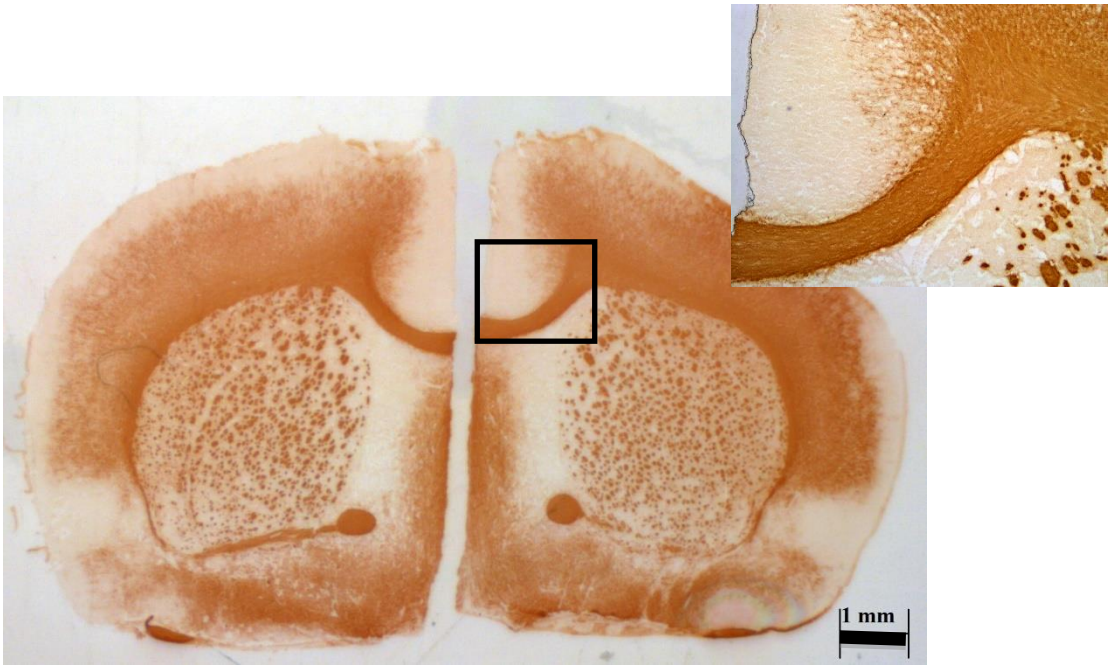


Figure 4.5. Brain sections stained with the immunohistochemical marker Olig2 to assess total number of oligodendroglial cells. Histological Olig2 stained sections at the region of the corpus callosum are presented (A). Oligodendroglia cells were counted using the Olig2 stain in two major white matter tracts, the corpus callosum (B) and Cingulum (C). No differences were found in the total number of Olig2 cells. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. Scale bars in (A): dashed bar = 200 μm and solid bars = 50 μm.

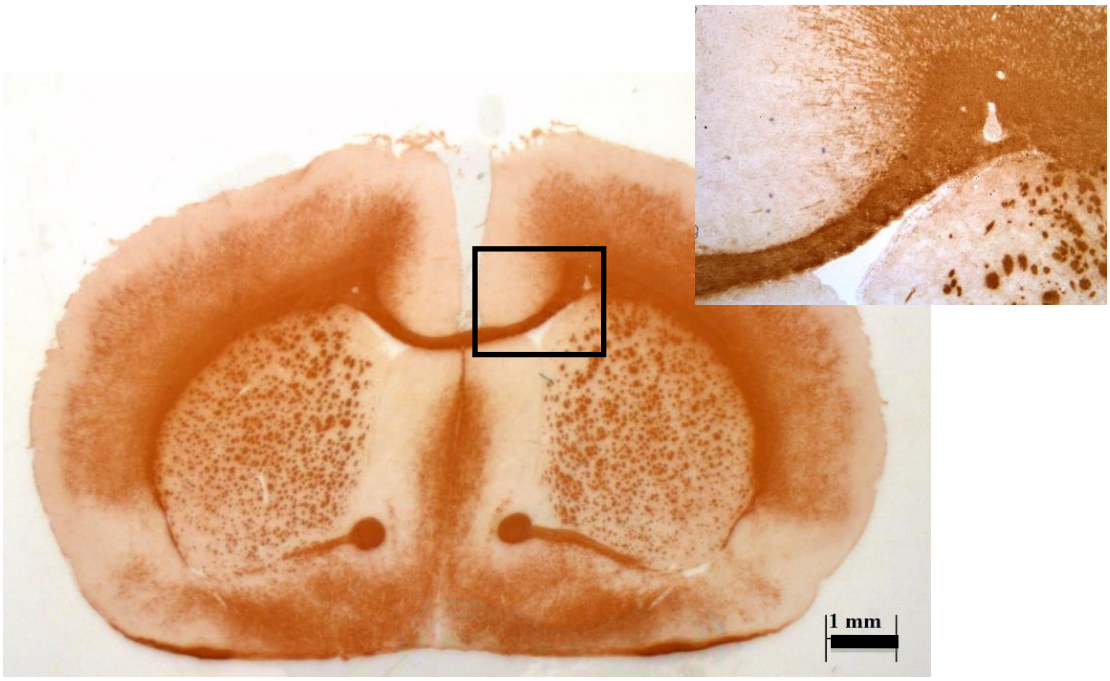
A)



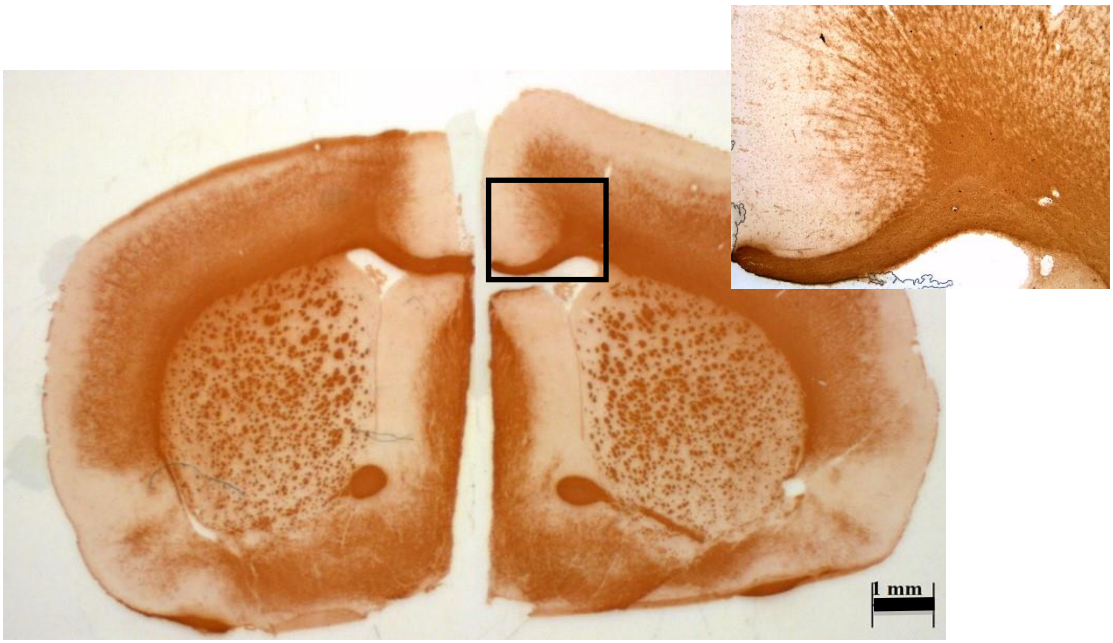
Saline



Saline + BrSp

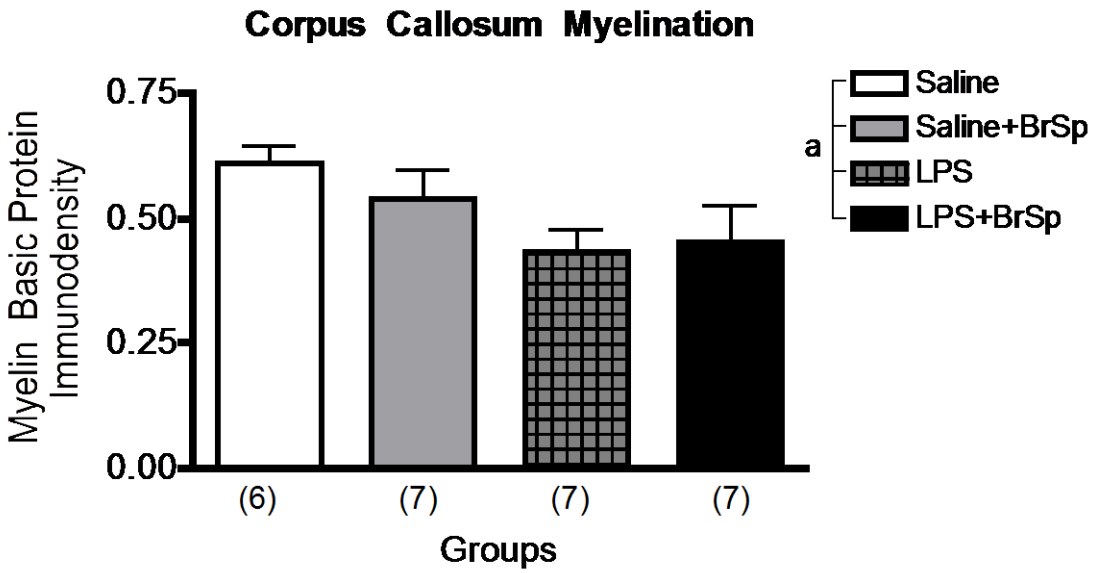


LPS



LPS + BrSp

B)



C)

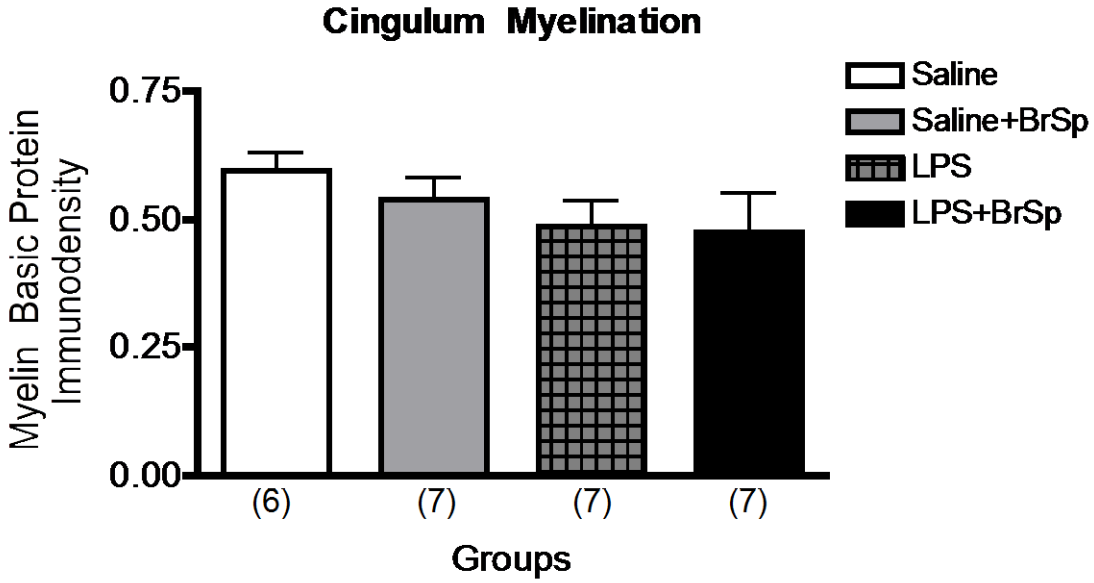


Figure 4.6. Myelin basic protein immunohistochemistry analyses for myelination. The immunodensity of myelin basic protein was evaluated on two major white matter tracts, the corpus callosum and the cingulum. A significant main effect of Treatment was found in the corpus callosum (A) where LPS and LPS + BrSp offspring had a reduced myelin basic protein

immunodensity. No differences were detected in the cingulum (B). Histological images of myelin basic protein (C). Inserts presented are the 5X magnification of the areas assessed. Data presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. Scale bars represent 1 mm.

4.2. OBJECTIVE 2

The next objective of the study was to identify possible mechanisms associated with the FIR model. In objective 1, white matter injury was identified, in agreement with the literature; however, no other gross abnormalities were observed. In addition to white matter injury, it was hypothesized that the inflammatory model also caused placental and fetal brain inflammation *in utero*, leading to abnormal supply of neurotrophic factors needed for proper brain maturation. Cytokines are also important for proper brain development; [98] both cytokines and neurotrophic factors interact to facilitate proper neuronal growth and development, and neurotrophic factors themselves are involved in neuronal development. [273-275] Aberrant neurotrophic factor support and a proinflammatory environment result in disturbed neuronal and oligodendroglial maturation, and sustain an activated gliosis response (astrogliosis and activated microglia). BrSp dietary consumption was hypothesized to prevent changes in the inflammatory response.

4.2.1. Placental and Fetal Weights, Viability, and Morphometrics

Following tissue extraction on E19 and E22, the weights of the placenta and fetus were recorded, and the crown rump length was measured. Sexes were not analyzed separately, as the pups were too young to sex by observation. No differences in placenta weight were observed at either time point (Figure 4.7A and 4.7B). Interestingly, there was a main effect of diet ($F(1,13)=9.6$, $p=0.008$) on fetal weights at E19 (Figure 4.7C). Saline + BrSp (1.7 ± 0.06g) and LPS + BrSp (1.7 ± 0.02g) weighed more than Saline (1.4 ± 0.07g) and LPS (1.6 ± 0.06g) fetuses. On E22, there was a main effect of treatment ($F(1,13)=31.0$, $p=0.0001$) on fetal weights (Figure

4.7D). LPS (4.6 ± 0.1g) and LPS + BrSp fetuses (4.7 ± 0.08g) weighed less than Saline (5.2 ± 0.1g) and Saline + BrSp fetuses (5.4 ± 0.2g). LPS fetuses were significantly lighter than Saline (Tukey's $p<0.05$) and Saline + BrSp (Tukey's $p<0.01$). LPS + BrSp fetuses were also significantly smaller than Saline + BrSp (Tukey's $p<0.01$). No differences in fetal to placenta weight ratio were detected on either E19 or E22 (Figure 4.7E and 4.7F).

On E19, no differences in the number of viable pups in the groups were detected (Figure 4.8A). On E22, a significant main effect of treatment ($F(1,13)=16.1$, $p=0.002$) was detected (Figure 4.8B). LPS (9.3 ± 1.3 pups) and LPS + BrSp (9.5 ± 1.5 pups) had fewer viable pups compared to Saline (14.3 ± 0.6 pups) and Saline + BrSp (14.0 ± 1.4 pups) litters. No effects on crown rump length were observed on E19 (Figure 4.8C). On E22, there was a significant main effect of treatment ($F(1,13)=25.4$, $p=0.0002$) (Figure 4.8D). LPS (4.5 ± 0.04 cm) and LPS + BrSp (4.5 ± 0.06 cm) fetuses were smaller in length compared to Saline (4.7 ± 0.05cm) and Saline + BrSp (4.8 ± 0.05cm). LPS fetuses were significantly smaller compared to Saline (Tukey's $p<0.05$) and Saline + BrSp (Tukey's $p<0.01$), and LPS + BrSp was significantly smaller compared to Saline + BrSp (Tukey's $p<0.05$).

4.2.2. Placental and Fetal Brain Cytokine mRNA Expression

The expression of cytokine mRNA on E19 and E22 in the placenta and fetal brains was assessed. IL-1β , TNF-α , IL-6, and IL-10 were evaluated for fold-expression changes compared to controls. On E19 in the placenta (Figure 4.9A), there was a significant main effect of treatment ($F(1,13)=12.4$, $p=0.003$) on IL-1β. LPS (4.1 ± 0.8 fold increase) and LPS + BrSp (6.2

± 2.1 fold increase) had a higher expression of IL-1β compared to Saline (1.1 ± 0.2 fold increase) and Saline + BrSp (1.2 ± 0.03 fold increase). Post hoc analyses detected a significant difference between Saline + BrSp and LPS + BrSp ($p<0.05$). A significant main effect of treatment was observed in TNF-α expression ($F(1,13)=19.9$, $p=0.0006$). LPS (6.8 ± 2.9 fold increase) and LPS + BrSp (7.9 ± 3.2 fold increase) had higher mRNA expression compared to Saline (0.8 ± 0.1 fold increase) and Saline + BrSp (1.1 ± 0.2 fold increase). There was a significant main effect of treatment on IL-6 ($F(1,13)=6.9$, $p=0.02$). LPS (20.5 ± 10.4 fold increase) and LPS + BrSp (26.5 ± 13.3 fold increase) had higher IL-6 mRNA expression compared to Saline (1.9 ± 0.8 fold increase) and Saline + BrSp (2.1 ± 1.2 fold increase). A significant main effect of treatment was detected in IL-10 expression ($F(1,12)=6.2$, $p=0.03$). There was higher IL-10 mRNA expression in the LPS (6.8 ± 2.5 fold increase) and LPS + BrSp (5.3 ± 1.4 fold increase) groups, compared to Saline (1.6 ± 0.3 fold increase) and Saline + BrSp (2.4 ± 0.8 fold increase).

Placental cytokine mRNA expression was also evaluated on E22 (Figure 4.9B). There were no effects of treatment or diet on IL-10, IL-1β or TNF-α mRNA expression. A significant main effect of diet ($F(1,13)=5.4$, $p=0.04$) on IL-6 was observed. There was lower expression of IL-6 in the Saline + BrSp (0.6 ± 0.2 fold increase) and LPS + BrSp (0.4 ± 0.06 fold increase) groups, compared to Saline (1.0 ± 0.2 fold increase) and LPS (1.0 ± 0.3 fold increase), although Tukey's test did not identify group differences.

In the brain, no significant changes were detected in the mRNA expression of IL-1β, TNF-α, and IL-6 at either E19 (Figure 4.9C) or E22 (Figure 4.9D). IL-1β has been correlated

with development of CP and neonatal encephalopathy, therefore a second probe spanning the exons that transcribe pro-IL-1β (exons 2,3 instead of 5,6) was employed. [276-278] A significant main effect of treatment ($F(1,12)=10.6$, $p=0.007$) and an interaction effect of treatment and diet ($F(1,12)=13.2$, $p=0.004$) were observed at E22 (Figure 4.9E). There was a trend towards a main effect of diet ($F(1,12)=4.7$, $p=0.05$). Brains from LPS fetuses had a significantly higher expression of the pro-IL-1β mRNA (8.4 ± 2.0 fold increase) compared to Saline (0.9 ± 0.4 fold increase, Tukey's $p<0.01$), Saline + BrSp (2.5 ± 0.5 fold increase, Tukey's $p<0.05$), and LPS + BrSp (2.1 ± 0.3 fold increase, Tukey's $p<0.01$). There was a significant interaction effect of treatment and diet ($F(1,12)=4.5$, $p=0.05$) on the anti-inflammatory cytokine IL-10 in the fetal brain at E19 (Figure 4.9C). LPS + BrSp (1.3 ± 0.6 fold increase) fetuses had a higher brain expression of IL-10 mRNA compared to Saline (0.8 ± 0.5 fold increase), Saline + BrSp (8.5×10^{-7} ± 5.1×10^{-7} fold increase) and LPS (0.2 ± 0.2 fold increase), although no group differences were identified. No significant differences were observed on E22.

Protein expression for the proinflammatory cytokines IL-1β, TNF-α, and IL-6 and the anti-inflammatory cytokine IL-10 was investigated in PD1 offspring brains. Studies have identified that upregulation or downregulation of either pro-inflammatory cytokines or anti-inflammatory cytokines can lead to behavioural deficits later in life, thus, the ratio of proinflammatory cytokines to IL-10 (IL-1β /IL-10, IL-6/IL-10, and TNF-α/IL-10) were assessed. [101] The ratio was evaluated to determine true upregulation or downregulation, since assessment of one pro-inflammatory cytokine without the anti-inflammatory cytokine may not represent true changes. Sex differences were identified therefore females (Figure 4.10A) and males (Figure 4.10B) were analyzed separately.

In the females (Figure 4.10A), no differences were detected in IL-1β /IL-10. Following analyses of TNF-α/IL-10, a significant main effect of Treatment (F(1,17)=8.4, p=0.01) was detected. The ratio of TNF-α/IL-10 was lower in the LPS (0.4 ± 0.03 protein ratio) and LPS + BrSp (0.4 ± 0.05 protein ratio) groups, compared to Saline (0.6 ± 0.04 protein ratio) and Saline + BrSp (0.5 ± 0.1 protein ratio). Similarly following IL-6/IL-10 analyses, a significant main effect of Treatment (F(1,16=11.1, p=0.004) was detected. LPS (10.0 ± 1.8 protein ratio) and LPS + BrSp (10.0 ± 1.8 protein ratio) groups had lower expression of IL-6/IL-10 compared to Saline (15.0 ± 1.2 protein ratio) and Saline + BrSp (13.2 ± 0.7 protein ratio).

Following analyses in the males (Figure 4.10B), no differences in IL-1β protein expression were observed. A significant interaction effect between treatment and diet (F(1,14)=8.0, p=0.01) was detected for TNF-α. Saline + BrSp pups had reduced expression of TNF-α/IL-10 (0.4 ± 0.03 protein ratio) compared to Saline (0.6 ± 0.05 fold increase, Tukey's $p<0.05$), LPS (0.4 ± 0.03 protein ratio), and LPS + BrSp (0.5 ±0.04 protein ratio). There was a significant interaction effect (F(1,14)=5.6, p=0.03) for IL-6/IL-10 protein expression. LPS + BrSp had a significantly higher expression of IL-6/IL-10 (13.5 ± 1.6 protein ratio) compared to Saline + BrSp (8.4 ± 1.1 protein ratio, Tukey's $p<0.05$).

4.2.3. Oxidative Stress

MMP-2 can be activated by pro-oxidant species, thus this can be used as a marker of oxidative stress (Figure 4.11A).[279,280] A significant main effect of sex (F(1,31)=8.5, p=0.007)

was detected, therefore males and females were separated prior to analyses (Figure 4.11B). Following separate analyses of males and females, no differences among the groups in MMP-2 were detected in either sex.

Next, the ratio of reduced to oxidized glutathione was investigated (Figure 4.11C). A significant main effect of sex was detected for reduced glutathione ($F(1,31)=8.9$, $p=0.006$), therefore males and females were analyzed separately. There was a significant main effect of diet ($F(1,13)=5.2$, $p=0.04$) and an interaction effect between treatment and diet ($F(1,13)=19.09$, $p=0.0008$) in females. LPS + BrSp pups had a significantly larger ratio of reduced to oxidized glutathione (4.6 ± 0.07) compared to Saline + BrSp (3.5 ± 0.1, Tukey's $p<0.01$) and LPS (3.3 ± 0.1, Tukey's $p<0.01$) pups. No significant differences were observed in males.

4.2.4. Neurotrophic Factors

Placental and fetal brain neurotrophic factor mRNA was analyzed. There were no detectable differences among the groups in BDNF, NGF, or NT-3 mRNA expression in the placenta on E19 (Figure 4.12A) or E22 (Figure 4.12B), nor in the offspring brain on E19 (Figure 4.12C). On E22, there were no differences observed for BDNF or NT-3 in the developing brain (Figure 4.12D); however, a significant main effect of treatment ($F(1,12)=34.5$, $p=0.0001$) and an interaction effect ($F(1,12)=12.8$, $p=0.004$) were detected for NGF (Figure 4.12D). The expression of NGF in the LPS (0.9 ± 0.05 fold increase) offspring and LPS + BrSp (0.7 ± 0.04 fold increase) was lower than in Saline (1.0 ± 0.02 fold increase) and Saline + BrSp (1.1 ± 0.06

fold increase) groups. These fold changes were significantly different between LPS + BrSp and Saline + BrSp (Tukey's, $p<0.001$).

4.2.5. Cell Death

Cell death was investigated on PD1. The initial examination of the apoptotic marker cleaved caspase-3 in the fetal brain (data not shown) did not show significant differences therefore, it was not pursued further. Fluoro-JadeB staining, which assessed degenerating neurons, was performed in several brain regions including the anterior commissure, corpus callosum, CA1 region of the hippocampus, thalamic nucleus reuniens, dorsal lateral septal nucleus, anteroventral and reticular thalamus nuclei, striatum, and the habenular nucleus. No differences were found (data not shown). Differences between sexes were not analyzed for cleaved caspase-3 and Fluoro-JadeB due to the small sample size.

NeuN, a marker of mature neurons, was used for quantification of neurons in several regions; anterior frontal cortex, hippocampus (CA1 and CA3), hypothalamus, caudate putamen, globus pallidus, posterior parietal cortex, and thalamus. A significant interaction effect was detected between diet and sex ($F(1,18)=7.06$, $p=0.02$) for the CA1 region, therefore, males and females were analyzed separately (Figure 4.13A). In the female group, there was a significant main effect of diet ($F(1,17)=6.6$, $p=0.02$). Saline + BrSp (90.2 ± 6.8 cells) and LPS + BrSp (87.0 ± 3.7 cells) offspring had fewer cells in the CA1 region compared to Saline (102.5 ± 5.1 cells) and LPS (101.8 ± 2.1). Tukey's test did not detect group differences. No differences were observed in the males. No other sex differences were detected therefore males and females were

grouped together in the remaining NeuN analyses. A significant main effect of diet ($F(1,26)=7.5$, $p=0.01$) was detected in the posterior parietal cortex (Figure 4.13B). Similar to the CA1 data, Saline + BrSp (47.3 ± 1.0 cells) and LPS + BrSp (49.8 ± 1.4 cells) pup brains had fewer cells compared to Saline (51.7 ± 1.0 cells) and LPS (54.2 ± 2.4 cells) pup brains, in the posterior parietal cortex. Tukey's test did not detect group differences. A significant interaction effect ($F(1,24)=6.6$, $p=0.02$) was detected in the anterior caudate putamen (Figure 4.13C). LPS + BrSp pups had higher cell numbers (59.1 ± 2.3 cells) compared to LPS (54.0 ± 2.1 cells). Saline (65.3 ± 4.0 cells) had significantly more NeuN positive cells compared to LPS (54.0 ± 2.1, Tukey's $p<0.05$). No differences were detected in the thalamus, globus pallidus, hypothalamus, CA3 of the hippocampus, or the anterior frontal cortex (data not shown).

4.2.6. Nrf2 Activation

Nrf2 was analyzed in nuclear extracts of the PD1 hemisphere. It was speculated that both LPS and BrSp would cause translocation of Nrf2 to the nucleus. However, no differences were detected in our studies (data not shown).

4.2.7. Neuronal Maturation

Changes to neuronal maturation were assessed on PD1, 7, and 21 with the following antibodies: growth associated protein 43 (neuronal growth cones), synaptophysin (synaptic vesicles), and syntaxin (pre-synaptic membrane). Sexes were not analyzed separately due to the small sample size. No significant effects on growth associated protein 43 were observed (Figure

4.14). Furthermore, no significant changes were associated with the synaptogenesis markers synaptophysin (Figure 4.15) and syntaxin (Figure 4.16) on PD7 or 21.

4.2.8. Oligodendroglial Maturation

We assessed maturation of oligodendrocytes using the markers Olig2 and 2',3'-cyclic-nucleotide 3'-phosphodiesterase at PD1, 7, and 21. Olig2 is a pan-oligodendroglial cell stain whereas 2',3'-cyclic-nucleotide 3'-phosphodiesterase characterizes oligodendroglial maturation and is involved in myelin production. Sexes were not analyzed separately due to small sample size. Not surprisingly and in agreement with our previous results, no significant differences were observed among the groups in Olig2 expression (Figure 4.17). No differences were observed in 2',3'-cyclic-nucleotide 3'-phosphodiesterase, a marker of mature oligodendrocytes on PD1 (Figure 4.18A) and 7 (Figure 4.18B); however, on PD21 in the white matter, there was a significant main effect of treatment ($F(1,25)=7.4$, $p=0.01$) (Figure 4.18C). The immunodensity in LPS (0.5±0.1 a.u.) and LPS + BrSp (0.6±0.09 a.u.) groups was lower than in Saline (0.9±0.07 a.u.) and Saline + BrSp (0.7±0.03 a.u.) groups. Furthermore, the LPS group had a significantly lower density compared to the Saline group (Tukey's $p<0.05$). Tukey's test did not detect a difference between LPS + BrSp in any of the three other groups. Thus, the data suggest that the FIR model induces a maturation deficit to the oligodendroglia population, impairing its ability to mature and produce myelin.

4.2.9. Gliosis

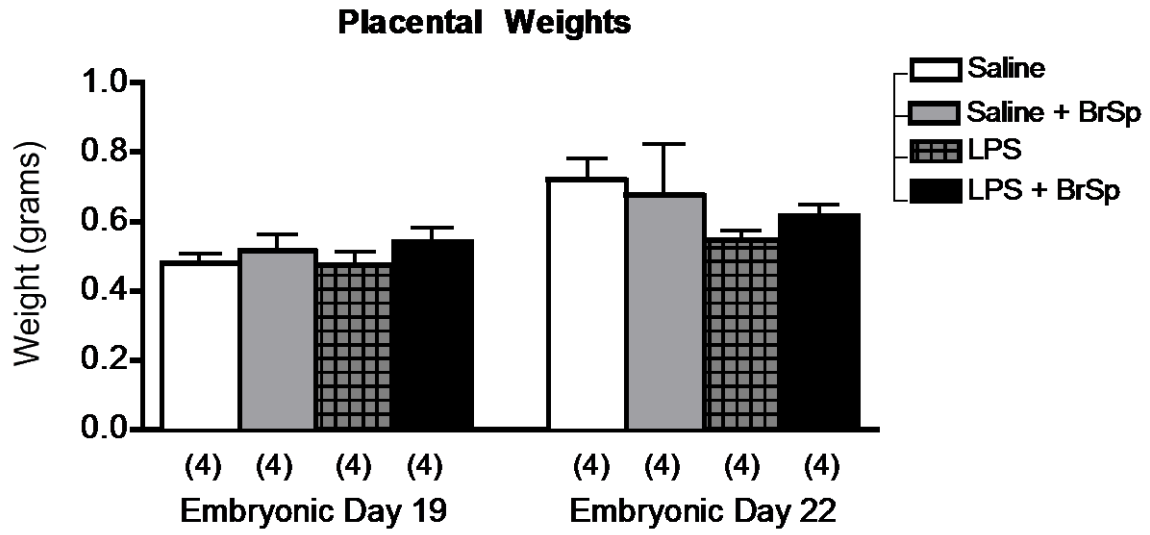
Astrogliosis was evaluated using the marker glial fibrillary acidic protein, however no differences among the groups were detected on PD1, 7, or 21 (Figure 4.19). Furthermore, activated microglia was evaluated on PD1; however, the data did not show any significant differences (data not shown). Sex differences were not analyzed due to small sample size. Overall, we did not detect activated astrocytes or microglia at these time points.

4.2.10. Summary

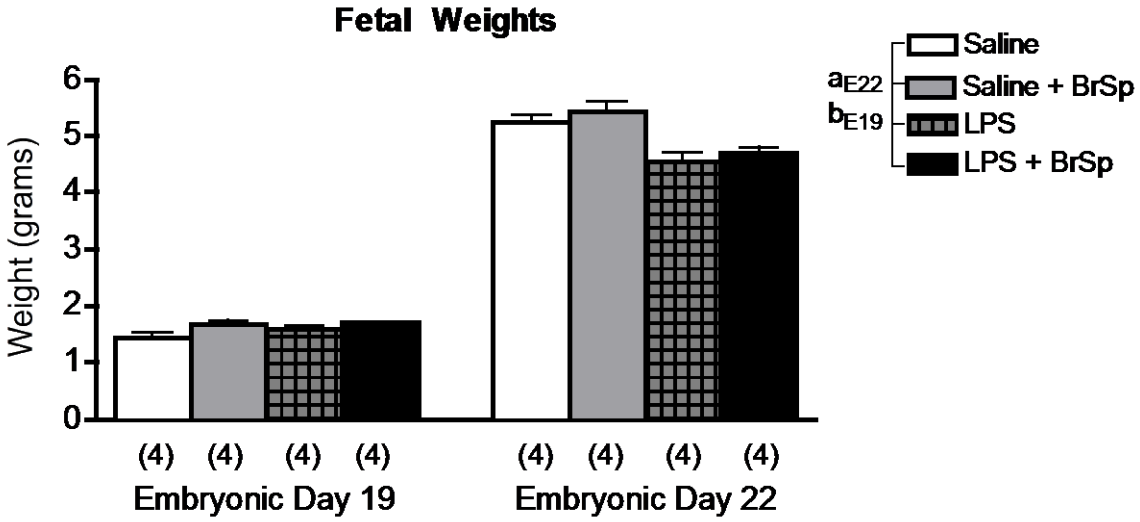
We observed an upregulation of proinflammatory cytokines on E19 in the placenta, and an increase of IL-10 on E19 and IL-1β on E22 in fetal brains. Changes to cytokine mRNA expression prompted investigation of neurotrophic factors as a possible link between inflammation and abnormal brain development. Neurotrophic factors were not altered in the placenta; however, a reduction in NGF was found in E22 fetal brains exposed to LPS. On PD1, there was a significantly higher ratio of reduced/oxidized glutathione in the LPS + BrSp newborn brains compared to other groups. Upon analyses of brain maturation, a decrease in 2',3'-cyclic-nucleotide 3'-phosphodiesterase expression was detected. We identified a lower number of NeuN cells in the CA1 region of the hippocampus and posterior parietal cortex in the Saline + BrSp and LPS + BrSp groups compared to the other groups. In addition there was a reduction in anterior caudate putamen NeuN cells in the LPS compared to Saline groups. No other differences were found. Thus, following *in utero* inflammation, a placental inflammatory response is induced, resulting in subsequent downstream increase in pro-IL-1β in the fetal brain. This

increase in cytokine exposure may cause a reduction in NGF. Alternatively, inflammatory cytokines can directly act on neurons and oligodendrocytes to induce these changes, working in parallel to the proposed signaling pathway.

A)


Placental Weights
Weight (grams)
1.0
0.8
0.6
0.4
0.2
0.0
(4) (4) (4) (4)
(4) (4) (4) (4)
Embryonic Day 19
Embryonic Day 22
Saline
Saline + BrSp
LPS
LPS + BrSp

B)


Fetal Weights
Weight (grams)
6
5
4
3
2
1
0
(4) (4) (4) (4)
(4) (4) (4) (4)
Embryonic Day 19
Embryonic Day 22
aE22
bE19
Saline
Saline + BrSp
LPS
LPS + BrSp

C)

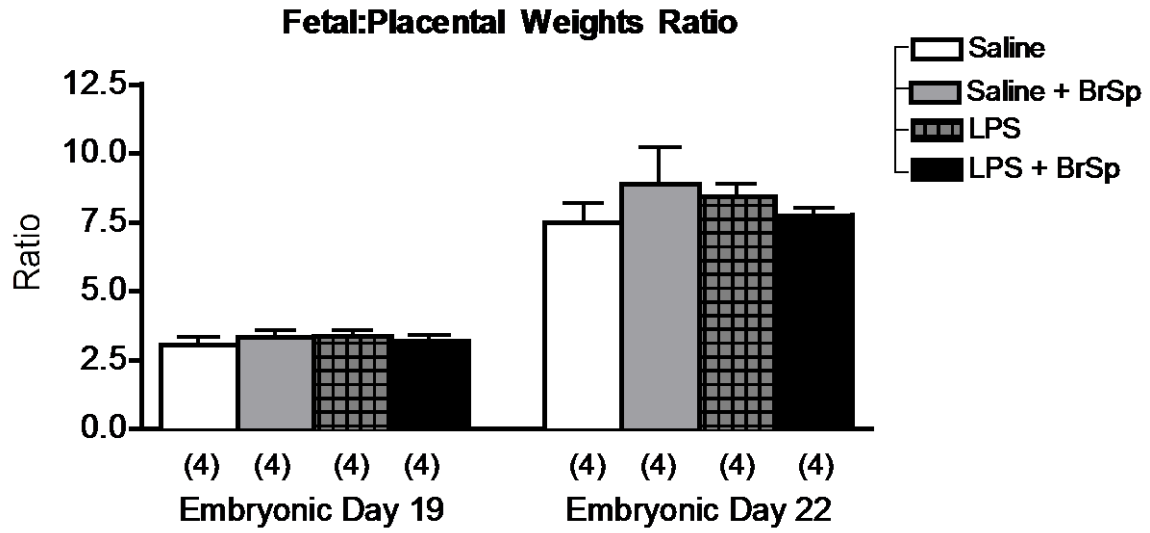
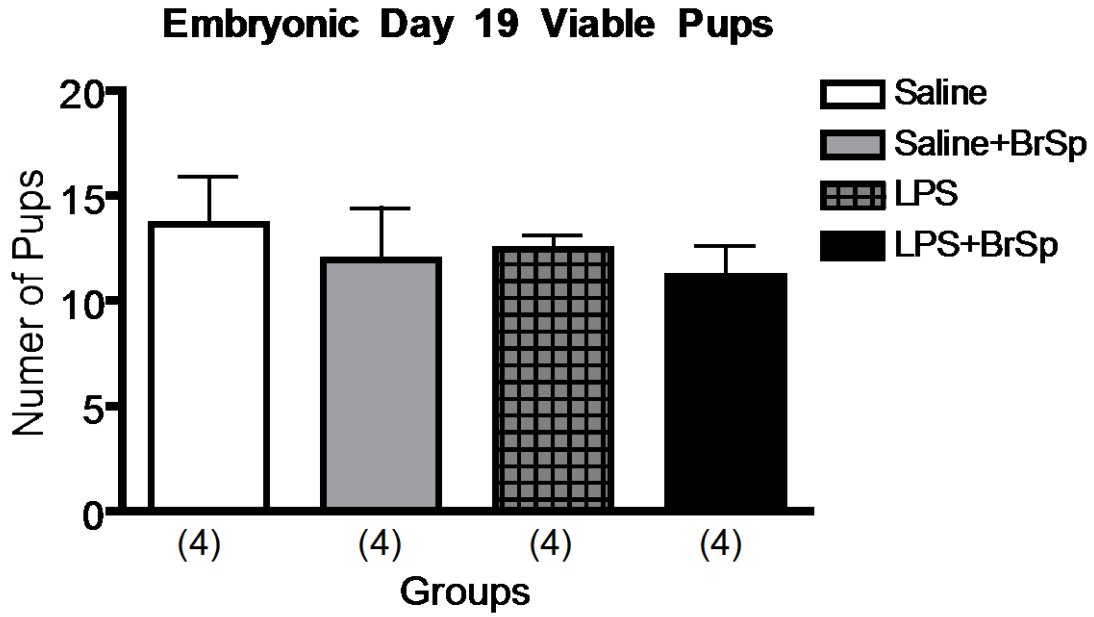
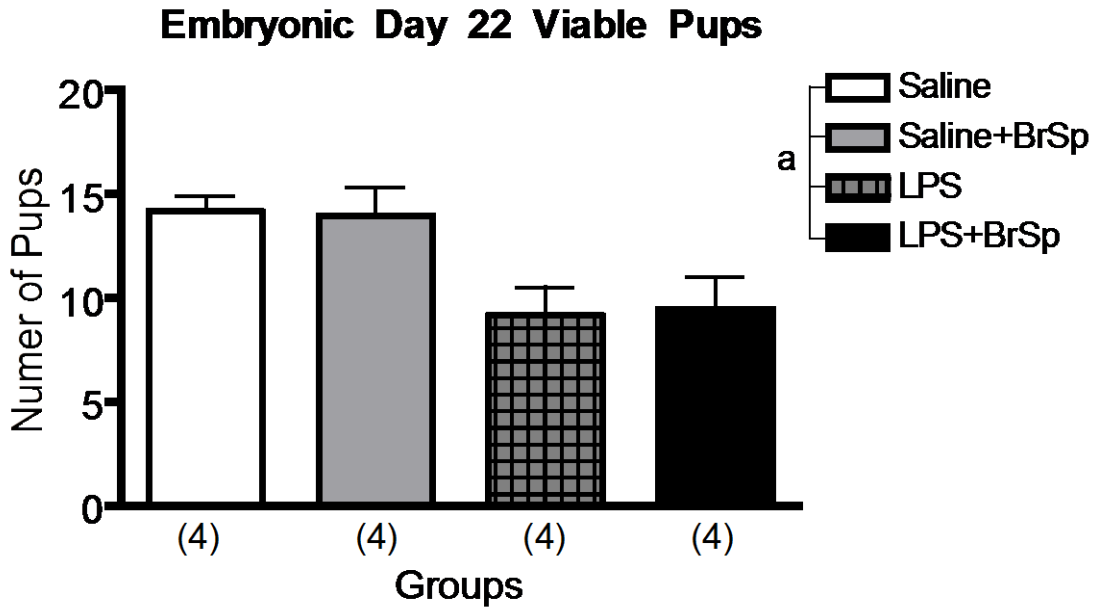


Figure 4.7. Physical biometric traits (weights) of E19 and E22 placentas and fetuses. No differences were detected in placental weights at E19 or E22 (A). On E19, there was a significant main effect of Diet on fetal weights (B). Saline + BrSp and LPS + BrSp weighed more than Saline and LPS fetuses. On E22, there was a significant main effect of Treatment on fetal weights (B). LPS and LPS + BrSp fetuses weighed less than Saline and Saline + BrSp fetuses. Analyses of fetal to placenta weight ratio on E19 and E22 (C) did not reveal any differences. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. b = significant main effect of Diet. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp).

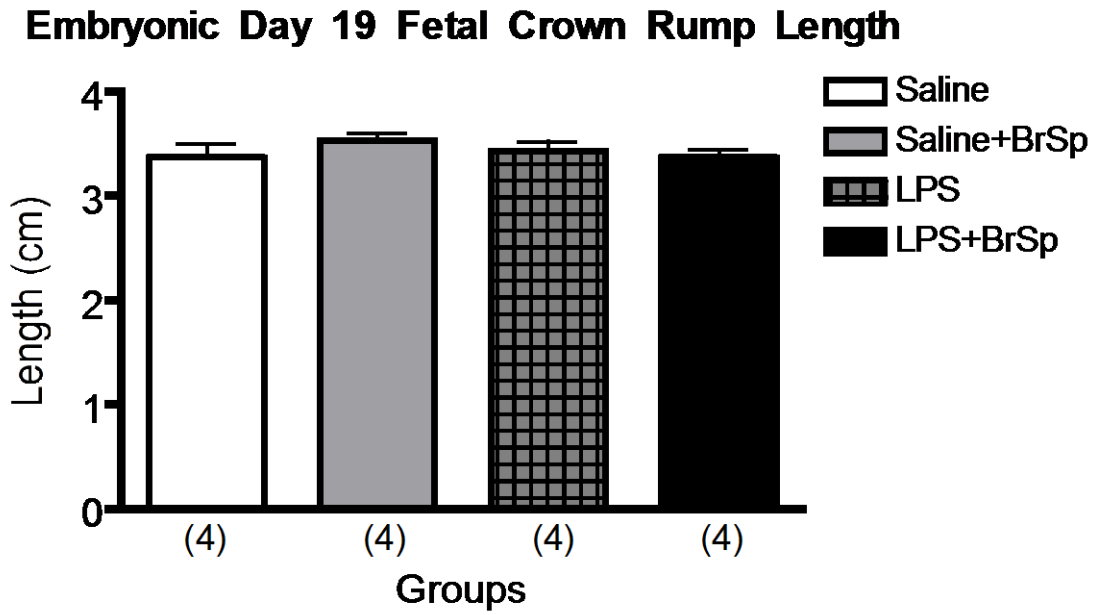
A)


Embryonic Day 19 Viable Pups
Numer of Pups
20
15
10
5
0
(4)
(4)
(4)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

B)


Embryonic Day 22 Viable Pups
Numer of Pups
20
15
10
5
0
(4)
(4)
(4)
(4)
Groups
a
Saline
Saline+BrSp
LPS
LPS+BrSp

C)



D)

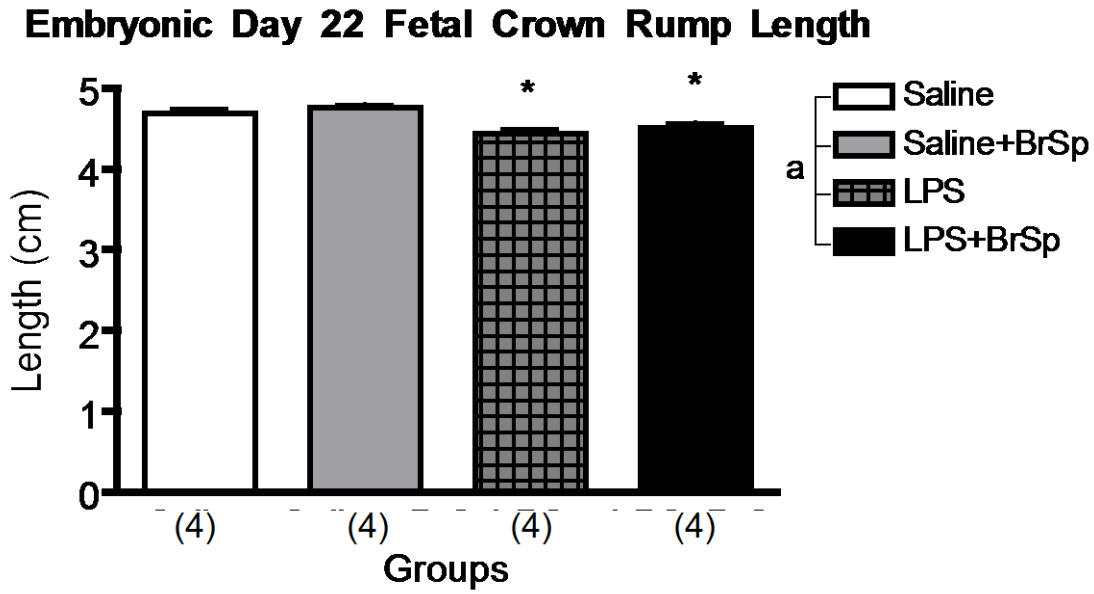
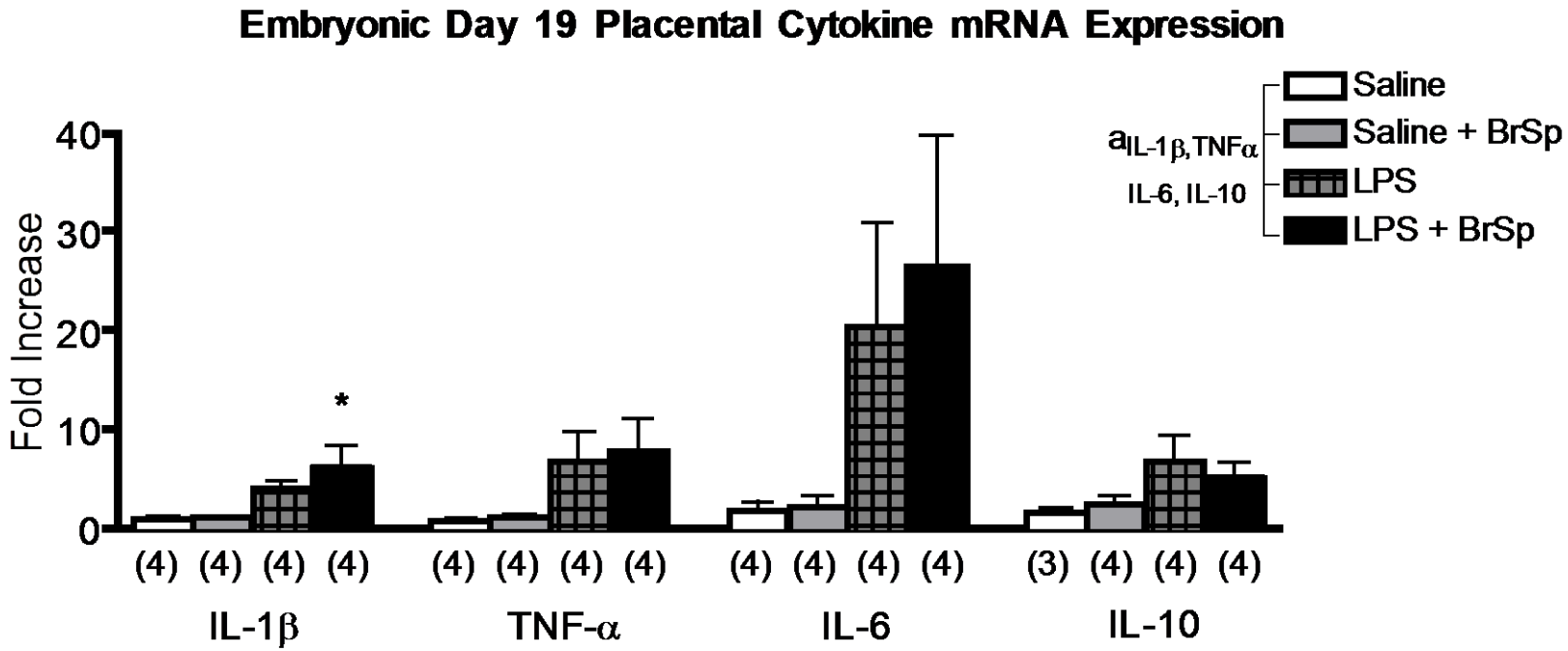


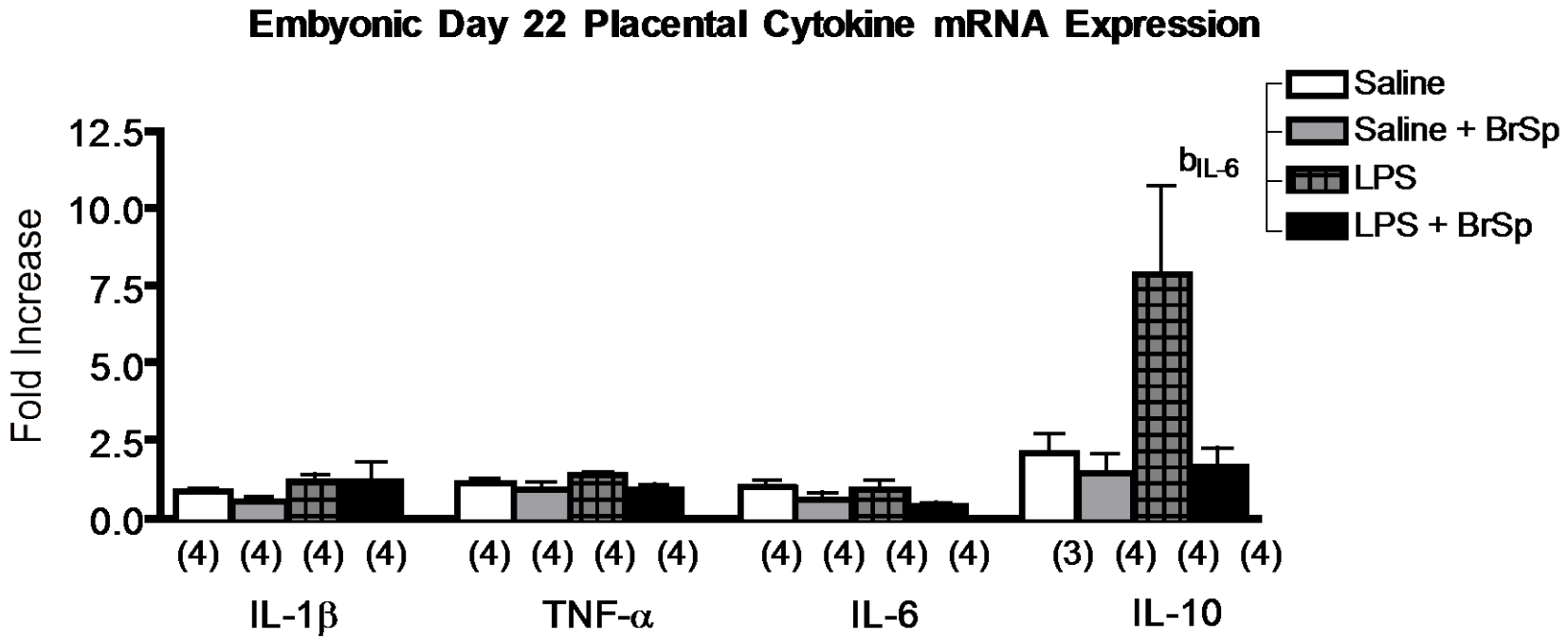
Figure 4.8. Fetal viability and morphometrics on E19 and 22. No differences were observed among the groups in the number of viable pups on E19 (A). On E22, a main effect of Treatment was detected on the number of viable pups (B). LPS and LPS + BrSp had fewer viable pups. On E19, no differences were detected in fetal crown rump length (C). On E22, a significant main effect of Treatment was detected in crown rump length (D). LPS and LPS + BrSp fetal crown

rump lengths were smaller than Saline and Saline + BrSp fetuses. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp).

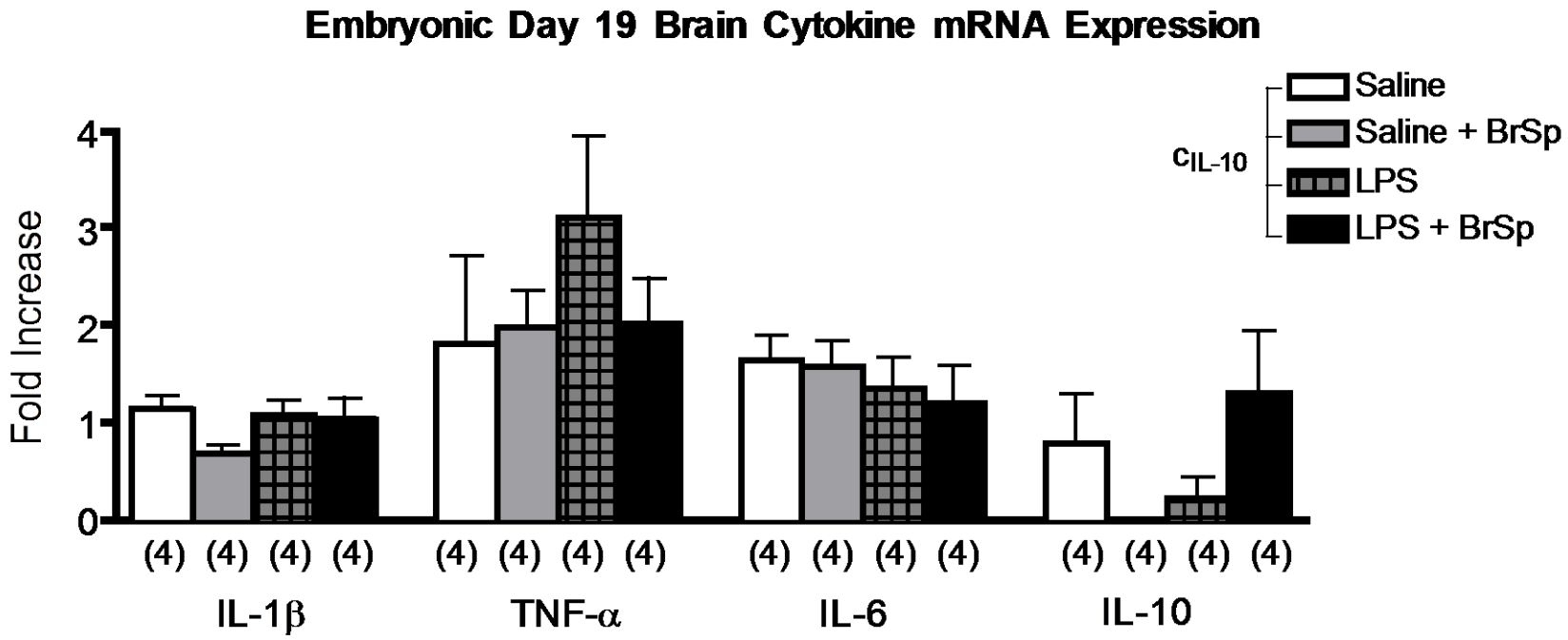
A)


Embryonic Day 19 Placental Cytokine mRNA Expression
Fold Increase
40
30
20
10
0
*
(4) (4) (4) (4)
(4) (4) (4) (4)
(4) (4) (4) (4)
(3) (4) (4) (4)
IL-1β
TNF-α
IL-6
IL-10
a IL-1β, TNFα
IL-6, IL-10
Saline
Saline + BrSp
LPS
LPS + BrSp

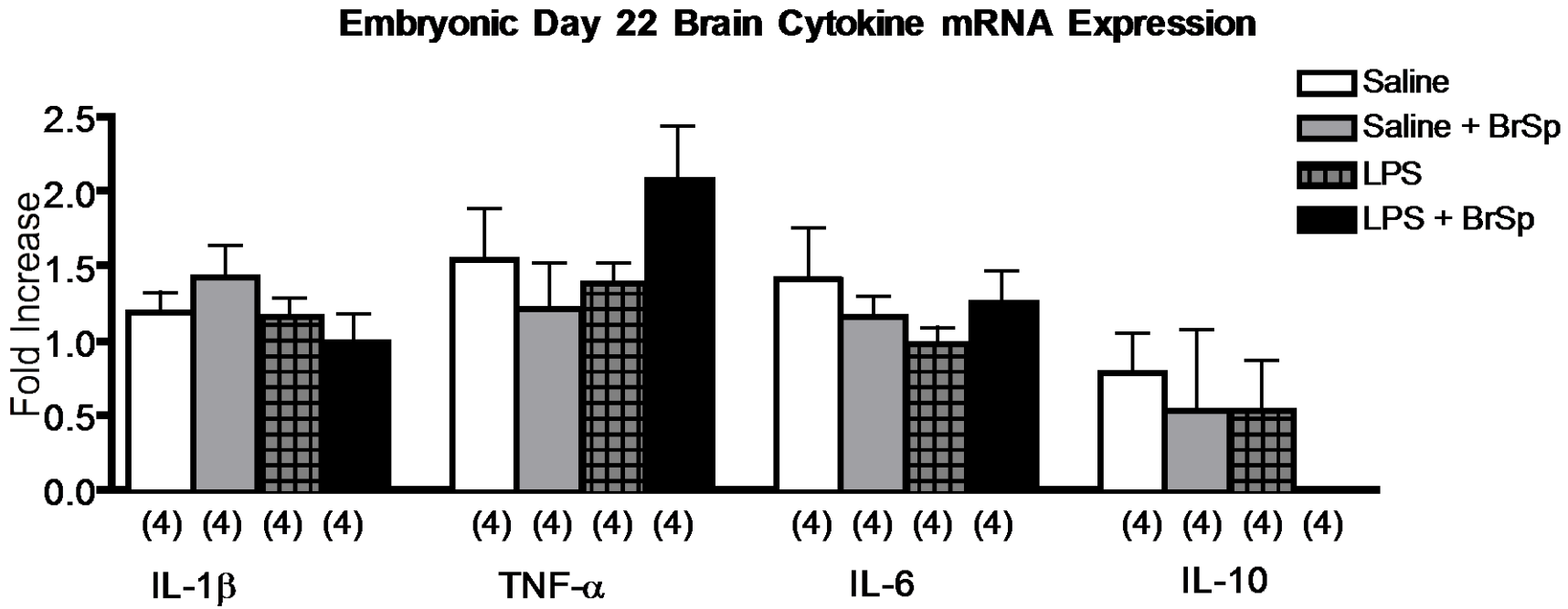
B)


Embyonic Day 22 Placental Cytokine mRNA Expression
Saline
Saline + BrSp
LPS
LPS + BrSp
Fold Increase
12.5
10.0
7.5
5.0
2.5
0.0
b IL-6
(4) (4) (4) (4) (4) (4) (4) (4) (4) (4) (4) (4) (3) (4) (4) (4)
IL-1β
TNF-α
IL-6
IL-10

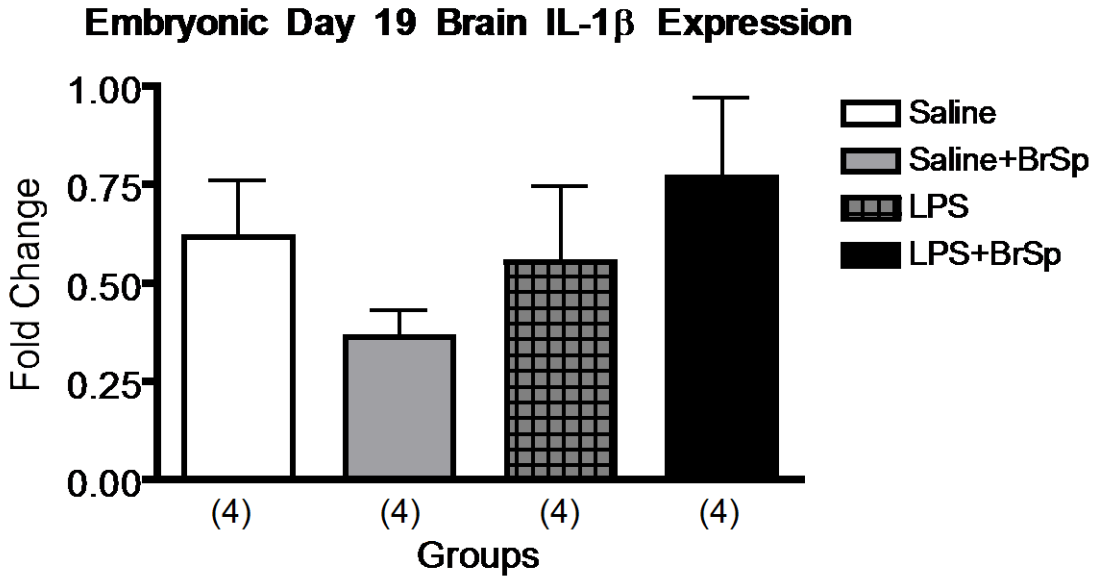
C)


Embryonic Day 19 Brain Cytokine mRNA Expression
Fold Increase
0
1
2
3
4
Saline
Saline + BrSp
LPS
LPS + BrSp
cIL-10
(4) (4) (4) (4)
IL-1β
(4) (4) (4) (4)
TNF-α
(4) (4) (4) (4)
IL-6
(4) (4) (4) (4)
IL-10

D)


Embryonic Day 22 Brain Cytokine mRNA Expression
Saline
Saline + BrSp
LPS
LPS + BrSp
Fold Increase
2.5
2.0
1.5
1.0
0.5
0.0
(4) (4) (4) (4)
(4) (4) (4) (4)
(4) (4) (4) (4)
(4) (4) (4) (4)
IL-1β
TNF-α
IL-6
IL-10

E)



F)

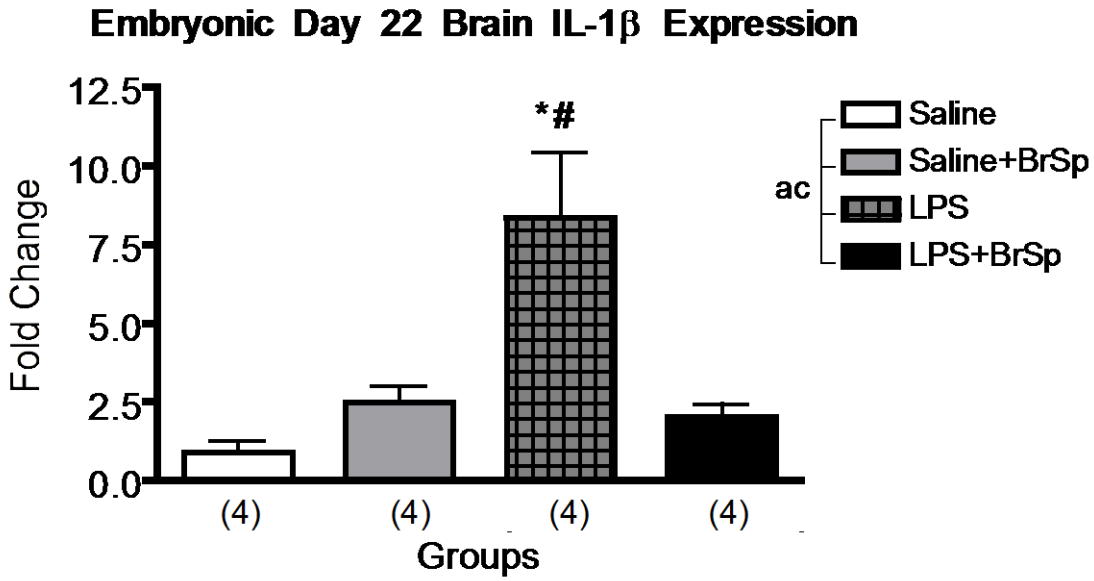
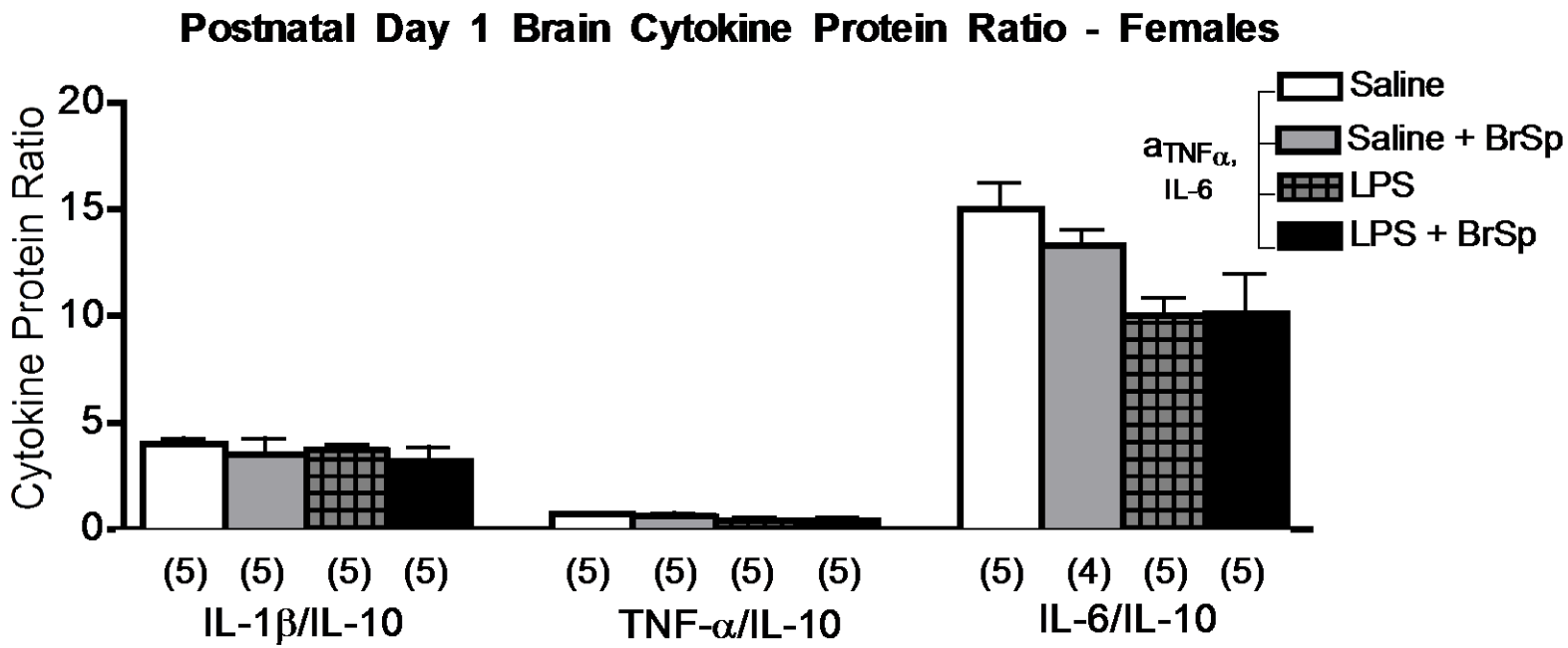


Figure 4.9. Placental and fetal cytokine mRNA expression on E19 and 22. Placental cytokine expression on E19 (A) was analyzed and a significant effect of Treatment was detected in all four cytokines. LPS and LPS + BrSp placentas had higher expression of all four cytokines compared to Saline and Saline + BrSp placentas. On E22 (B), a significant main effect of Diet

was detected for IL-6. Saline + BrSp and LPS + BrSp placentas had a reduced expression of IL-6, compared to other groups; no other differences were detected. Fetal brain analyses on E19 (C) did not detected any differences in fold expression of pro-inflammatory cytokines. However, a significant interaction effect was found where LPS + BrSp had higher IL-10. On E22 (D), no differences were detected. When probing for IL-1β spanning a different exon, no differences were detected on E19 (E), however, a significant main effect of Treatment and an interaction was detected on E22 (F). Fetuses exposed to LPS had a significant increase in brain IL-1β mRNA expression compared to Saline, Saline + BrSp, and LPS + BrSp fetal brains. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. b = significant main effect of Diet. c= significant interaction effect between Treatment and Diet. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp). # = significant differences between LPS and LPS + BrSp.

A)


Postnatal Day 1 Brain Cytokine Protein Ratio - Females
Cytokine Protein Ratio
20
15
10
5
0
Saline
Saline + BrSp
LPS
LPS + BrSp
aTNFα, IL-6
(5) (5) (5) (5)
IL-1β/IL-10
(5) (5) (5) (5)
TNF-α/IL-10
(5) (4) (5) (5)
IL-6/IL-10

B)

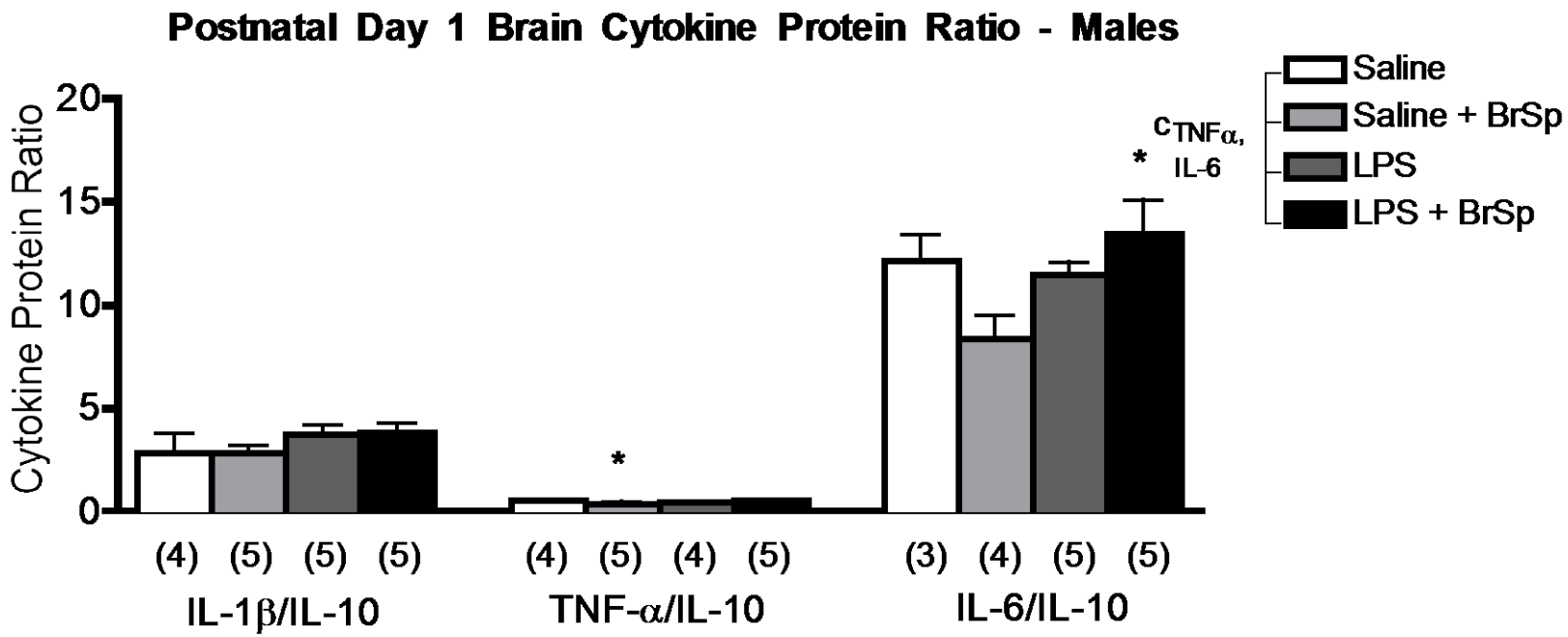
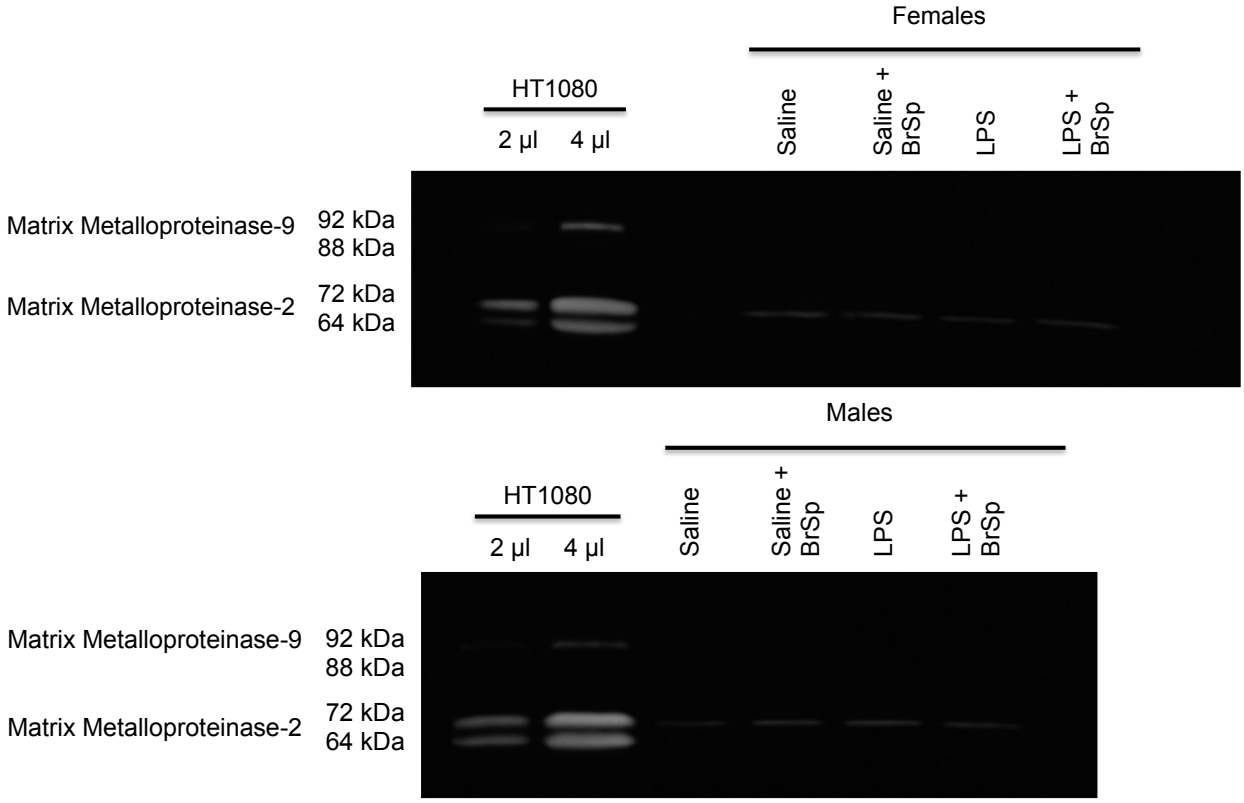
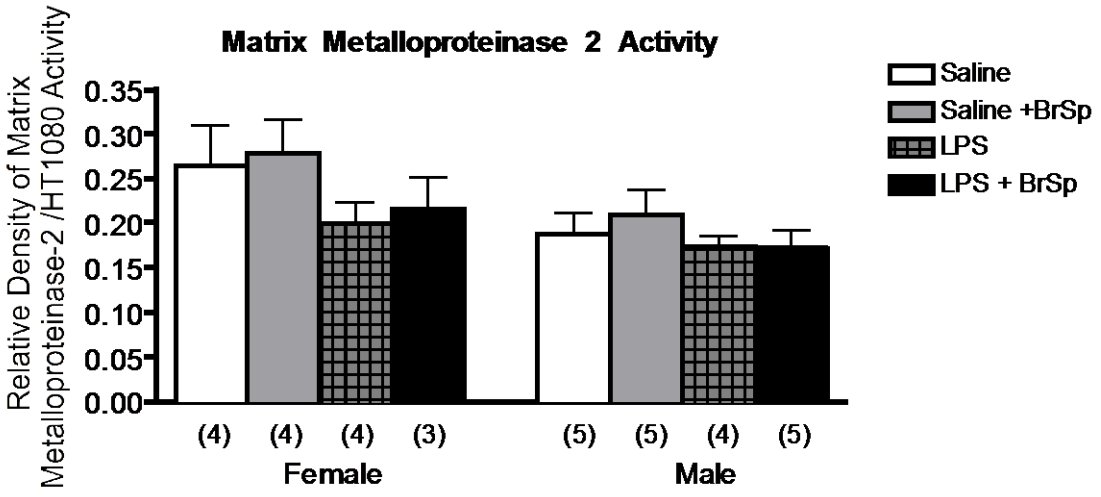

Postnatal Day 1 Brain Cytokine Protein Ratio - Males
Cytokine Protein Ratio
20
15
10
5
0
Saline
Saline + BrSp
LPS
LPS + BrSp
cTNFα, IL-6
*
*
(4) (5) (5) (5)
(4) (5) (4) (5)
(3) (4) (5) (5)
IL-1β/IL-10
TNF-α/IL-10
IL-6/IL-10

Figure 4.10. The ratio of pro-inflammatory cytokine protein expression (Il-1β, TNF-α, and IL-6) to the anti-inflammatory cytokine (IL-10) on PD1 rat pup brains. In females, no differences were detected in IL-1β. A significant main effect of treatment was detected for TNF-α and IL-6, where both LPS and LPS + BrSp pups had lower expression of TNF-α/IL-10 and IL-6/IL-10. In the males, a significant interaction effect was detected for TNF-α and IL-6. Saline + BrSp had the lowest expression of the TNF-α/IL-10 and IL-6/IL-10 compared to the other groups. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. c= significant interaction effect between Treatment and Diet. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp

A)


Females
HT1080
2 µl
4 µl
Saline
Saline + BrSp
LPS
LPS + BrSp
Matrix Metalloproteinase-9
92 kDa
88 kDa
Matrix Metalloproteinase-2
72 kDa
64 kDa
Males
HT1080
2 µl
4 µl
Saline
Saline + BrSp
LPS
LPS + BrSp
Matrix Metalloproteinase-9
92 kDa
88 kDa
Matrix Metalloproteinase-2
72 kDa
64 kDa

B)


Matrix Metalloproteinase 2 Activity
Relative Density of Matrix Metalloproteinase-2 /HT1080 Activity
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
(4) (4) (4) (3)
Female
(5) (5) (4) (5)
Male
Saline
Saline +BrSp
LPS
LPS + BrSp

C)

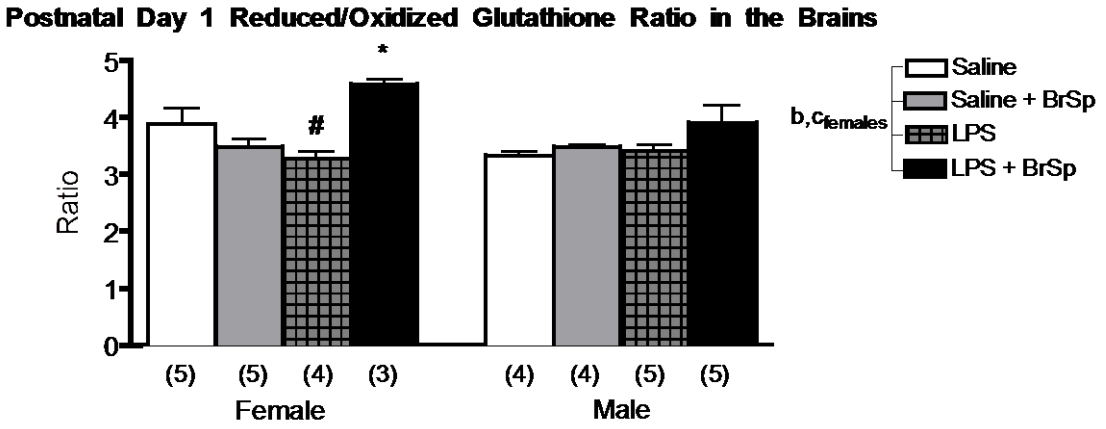
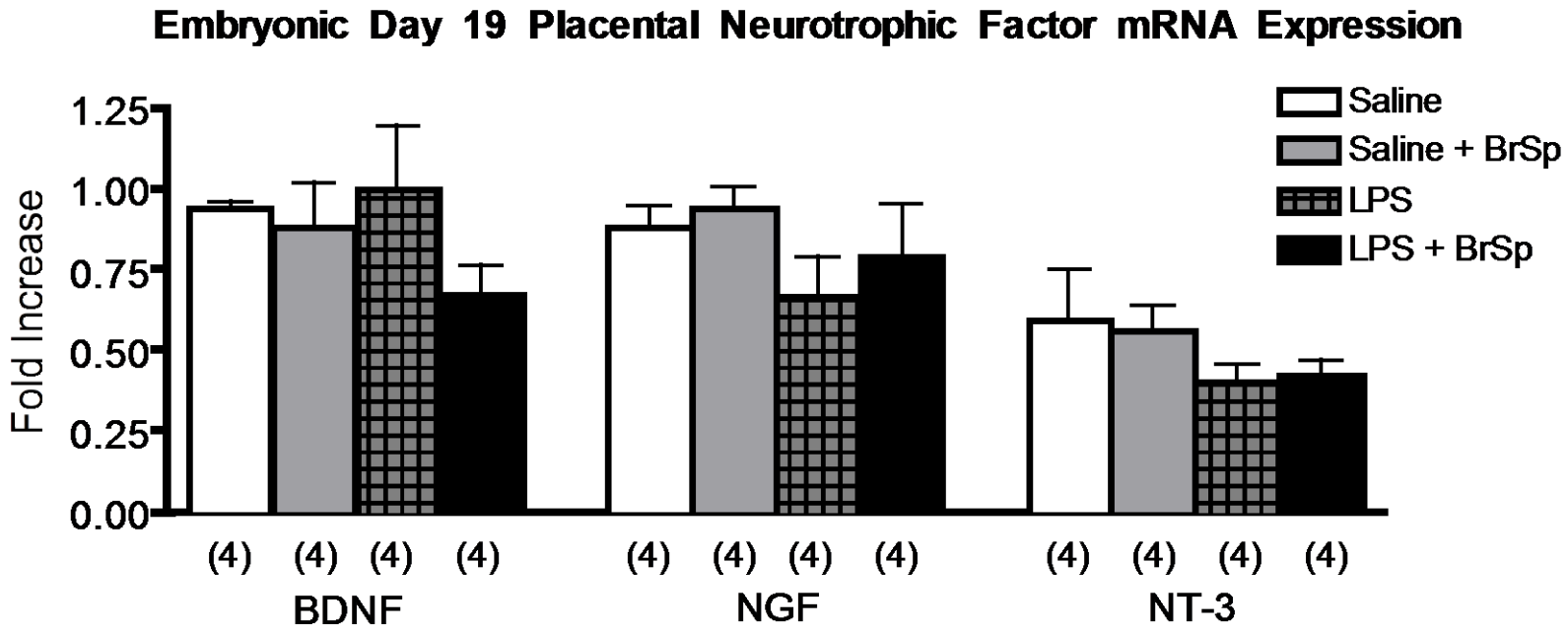
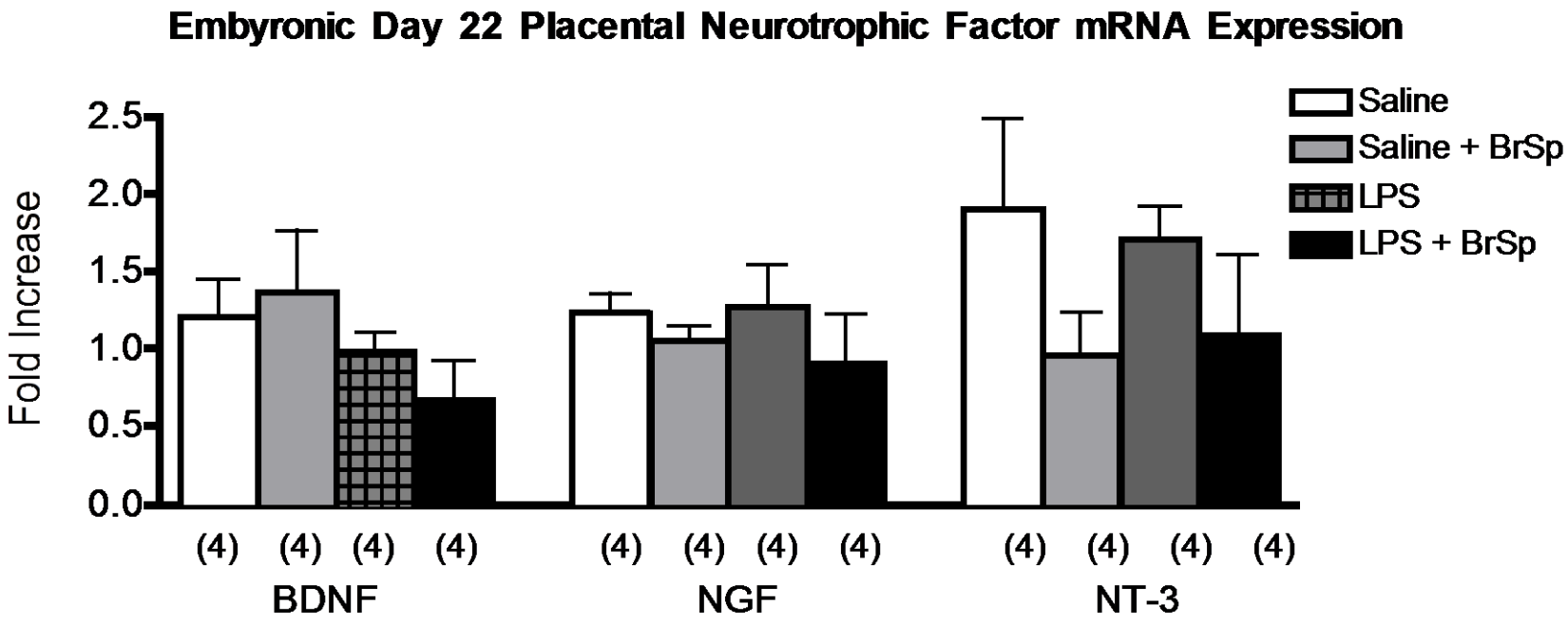


Figure 4.11. MMP-2 and the reduced/oxidized glutathione ratio assessed as indices of oxidative stress in the PD1 rat pup brains. MMP-2 activity was measured using gelatin zymography and the conditioned media from HT1080 cells were used as a control (A). Following MMP-2 analyses (B), no differences were detected. Upon assessment of glutathione, there was a significant main effect of Diet and an interaction effect between Treatment and Diet in the females. LPS + BrSp pups had a higher brain expression of reduced/oxidized glutathione compared to Saline + BrSp and LPS pup brains. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. b = significant main effect of Diet. c= significant interaction effect between Treatment and Diet. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp). # = significant differences between LPS and LPS + BrSp.

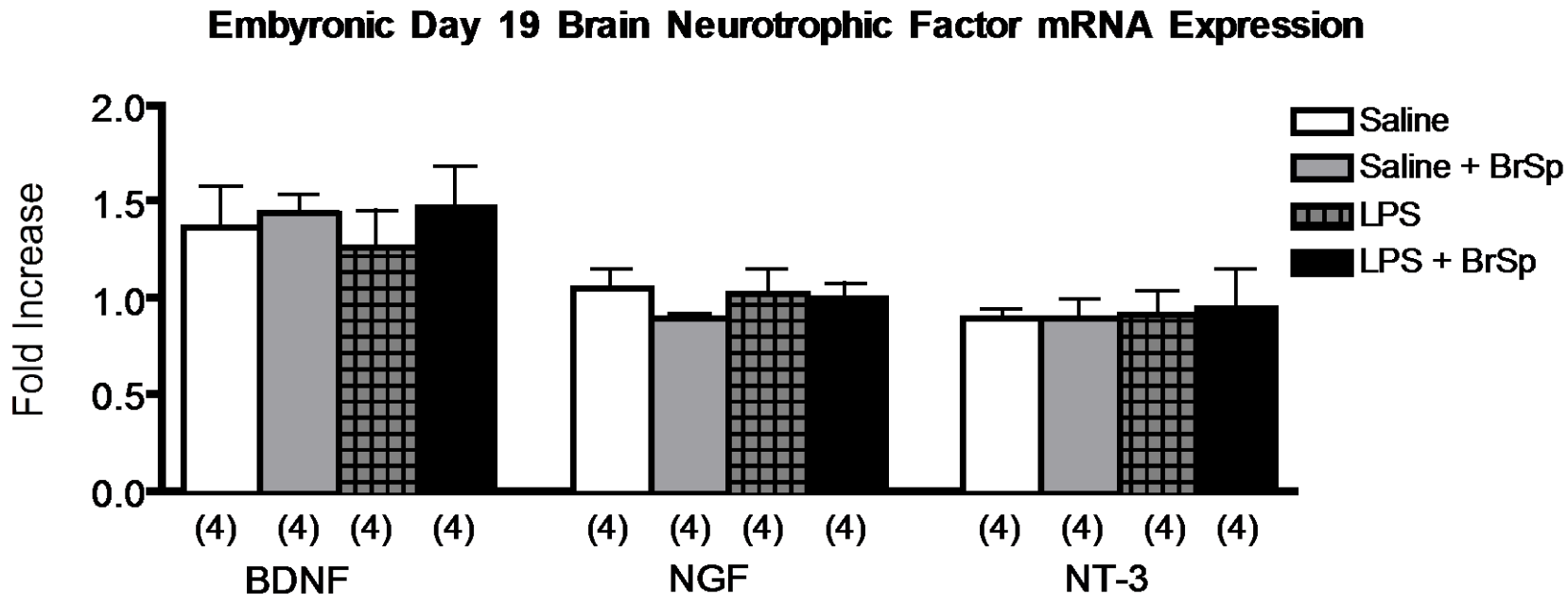
A)


Embryonic Day 19 Placental Neurotrophic Factor mRNA Expression
Fold Increase
1.25
1.00
0.75
0.50
0.25
0.00
Saline
Saline + BrSp
LPS
LPS + BrSp
(4) (4) (4) (4)
BDNF
(4) (4) (4) (4)
NGF
(4) (4) (4) (4)
NT-3

B)


Embyronic Day 22 Placental Neurotrophic Factor mRNA Expression
Fold Increase
2.5
2.0
1.5
1.0
0.5
0.0
Saline
Saline + BrSp
LPS
LPS + BrSp
(4) (4) (4) (4)
BDNF
(4) (4) (4) (4)
NGF
(4) (4) (4) (4)
NT-3

C)


Embyronic Day 19 Brain Neurotrophic Factor mRNA Expression
Fold Increase
2.0
1.5
1.0
0.5
0.0
Saline
Saline + BrSp
LPS
LPS + BrSp
(4) (4) (4) (4)
BDNF
(4) (4) (4) (4)
NGF
(4) (4) (4) (4)
NT-3

D)

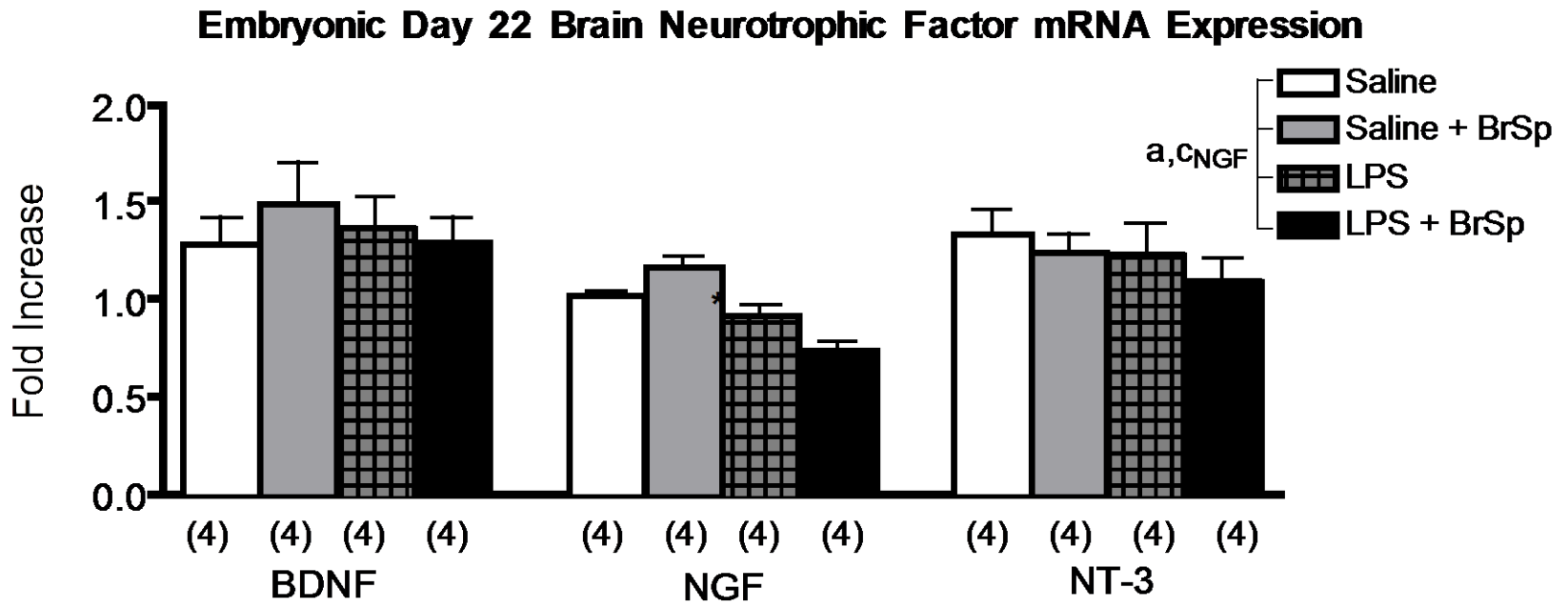
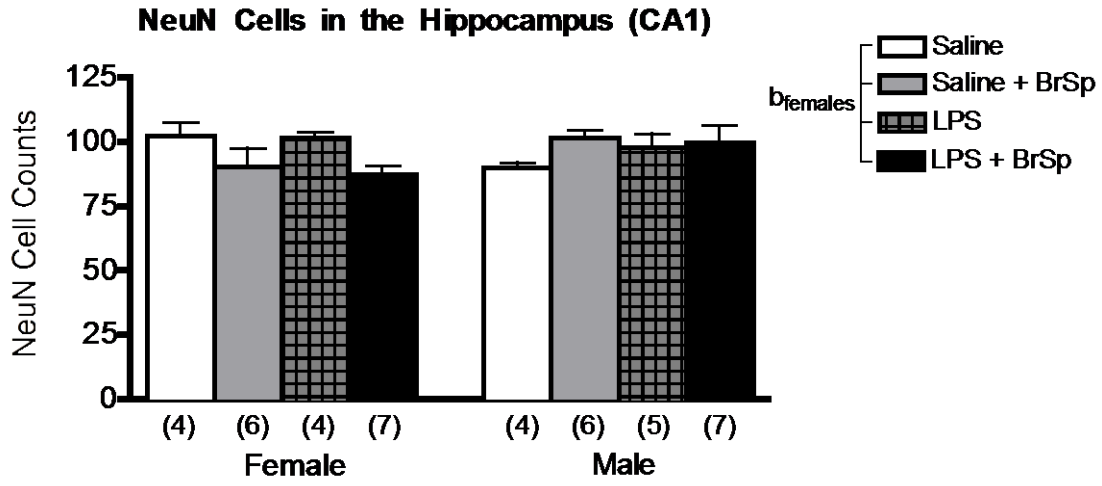
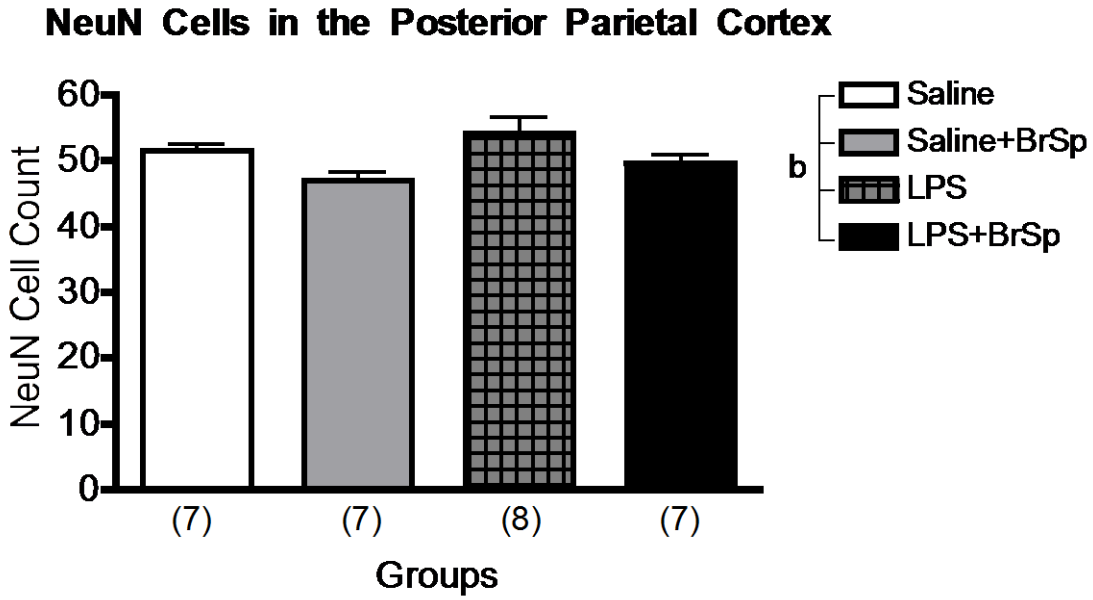

Embryonic Day 22 Brain Neurotrophic Factor mRNA Expression
Fold Increase
2.0
1.5
1.0
0.5
0.0
Saline
Saline + BrSp
LPS
LPS + BrSp
a,cNGF
*
(4) (4) (4) (4)
BDNF
(4) (4) (4) (4)
NGF
(4) (4) (4) (4)
NT-3

Figure 4.12. The mRNA expression of neurotrophic factors BDNF, NGF, and NT-3 in the placenta and fetal brains on E19 and E22. No differences were detected in the fetal brain on E19 (C). On E22, no differences were detected in the fetal brain in BDNF or NT-3 mRNA expression (D). A significant main effect of Treatment and an interaction effect were detected following NGF analyses on E22 (D). LPS and LPS + BrSp had a reduced expression of NGF compared to Saline and Saline + BrSp. The decreased expression in the LPS + BrSp group was significantly different from Saline + BrSp. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a = significant main effect of Treatment. c = significant interaction effect between Treatment and Diet. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp).

A)


NeuN Cells in the Hippocampus (CA1)
NeuN Cell Counts
125
100
75
50
25
0
(4) (6) (4) (7) (4) (6) (5) (7)
Female
Male
b females
Saline
Saline + BrSp
LPS
LPS + BrSp

B)


NeuN Cells in the Posterior Parietal Cortex
NeuN Cell Count
60
50
40
30
20
10
0
(7) (7) (8) (7)
Groups
b
Saline
Saline+BrSp
LPS
LPS+BrSp

C)

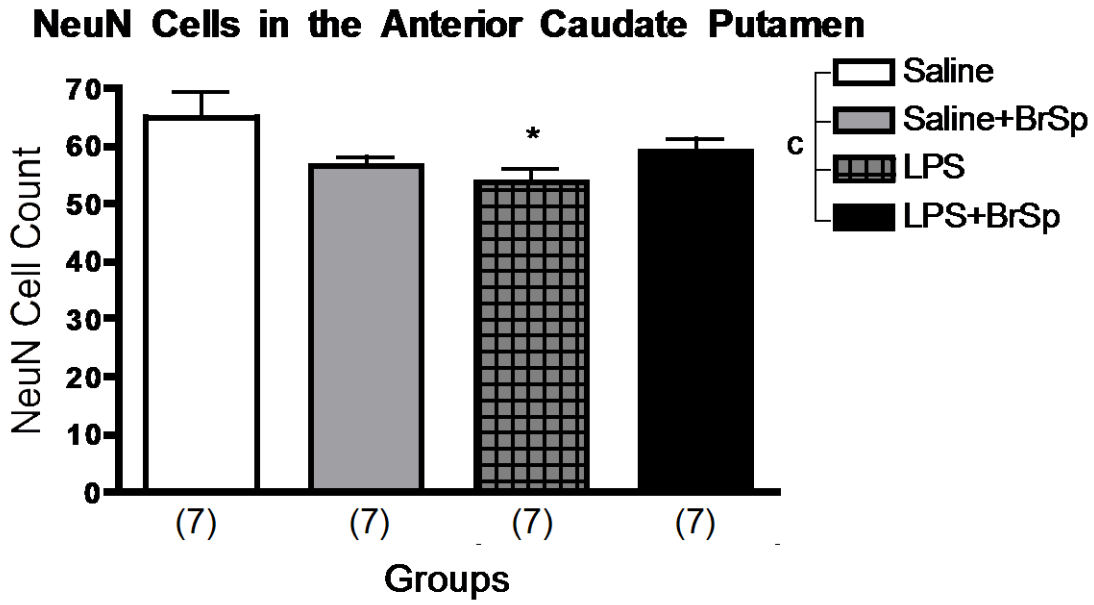
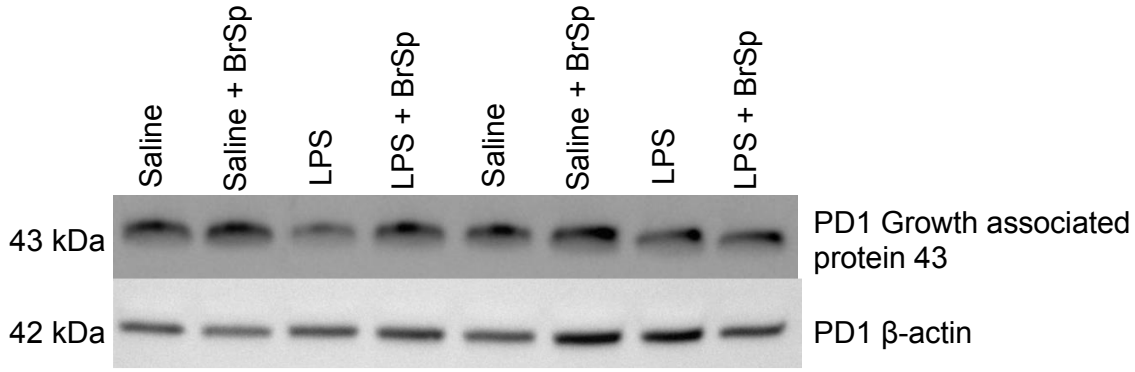
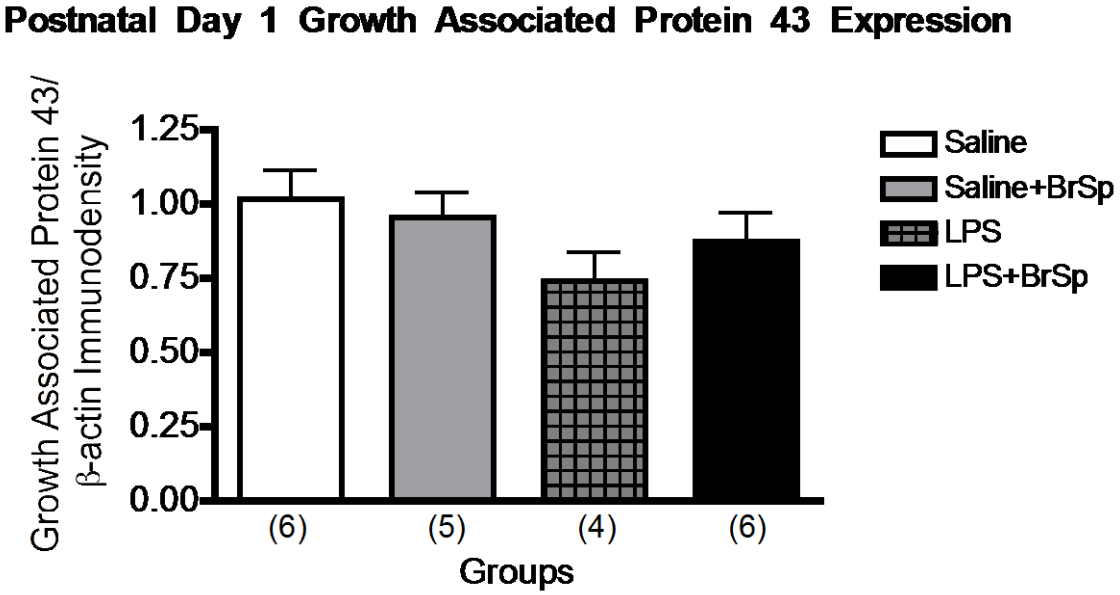


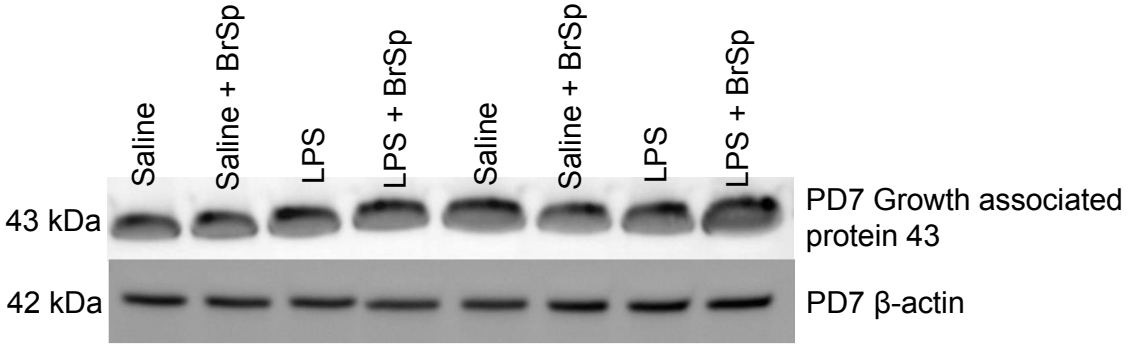
Figure 4.13. Number of mature neuronal cells counted using the immunohistochemical marker NeuN. In the CA1 region of the hippocampus (A) and the posterior parietal cortex (B), a significant main effect of Diet was detected in female pups. Saline + BrSp and LPS + BrSp groups had fewer NeuN positive cells compared to Saline and LPS. In the anterior caudate putamen (C), a significant interaction effect was found. Tukey's post hoc test identified significantly fewer NeuN positive cells in the LPS group compared to Saline. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. c= significant interaction effect between Treatment and Diet. * = significant difference between respective control (i.e. Saline vs LPS or Saline + BrSp vs LPS + BrSp).

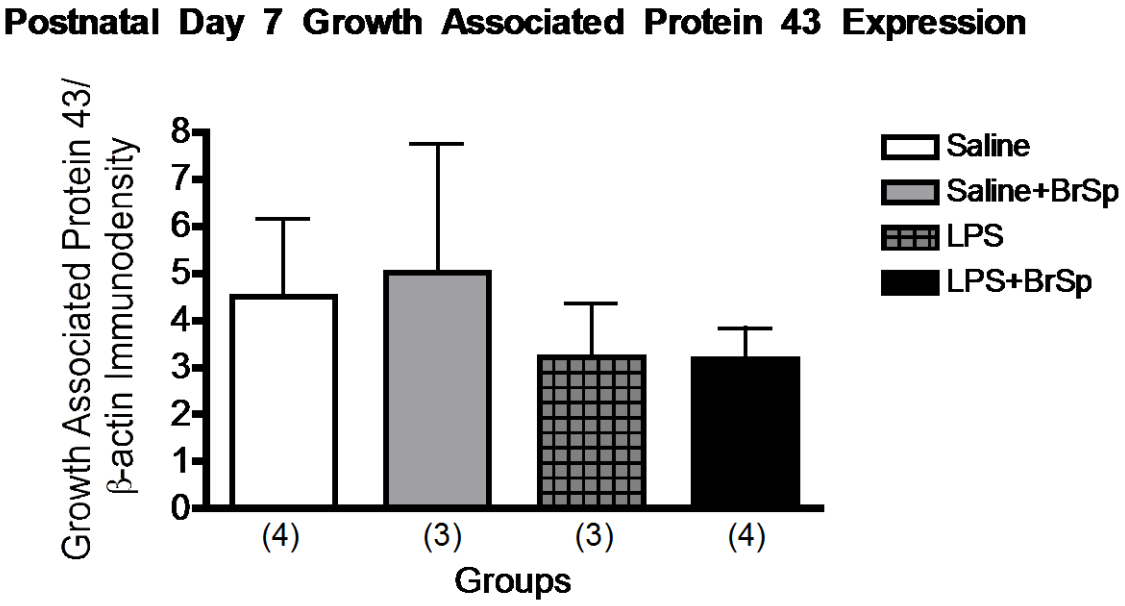
A)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
43 kDa
42 kDa
PD1 Growth associated protein 43
PD1 β-actin

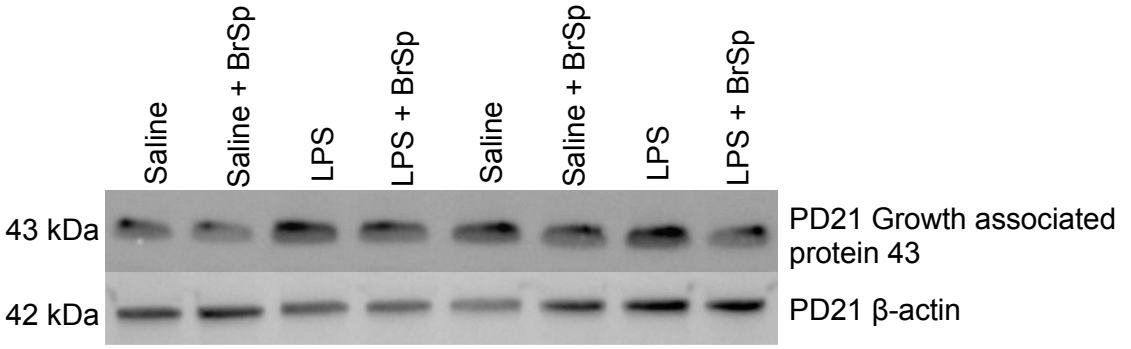

Postnatal Day 1 Growth Associated Protein 43 Expression
Growth Associated Protein 43/ β-actin Immunodensity
1.25
1.00
0.75
0.50
0.25
0.00
(6)
(5)
(4)
(6)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

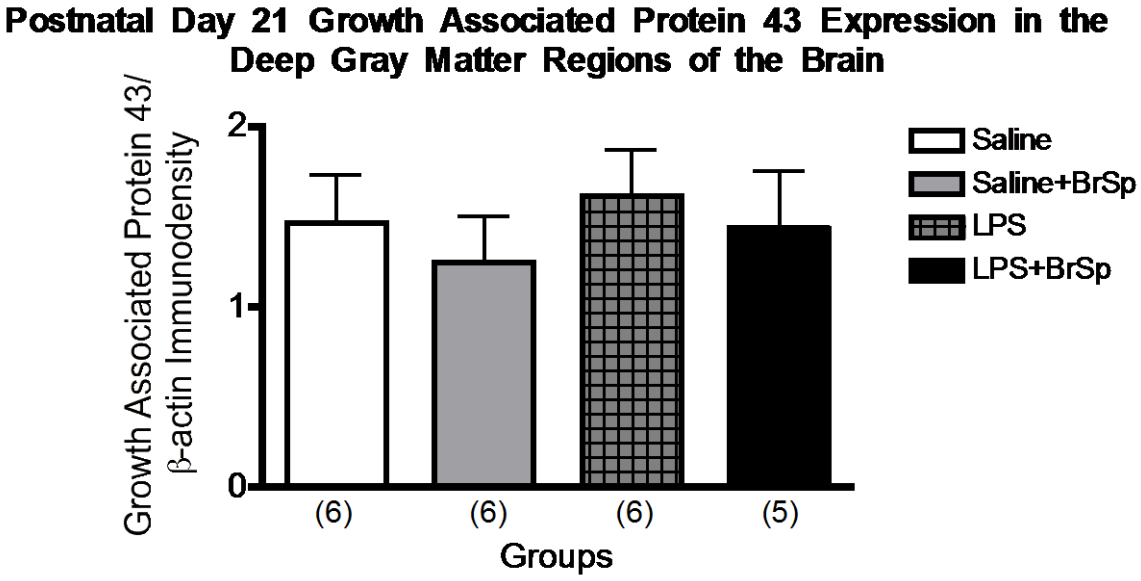
B)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
43 kDa
42 kDa
PD7 Growth associated protein 43
PD7 β-actin

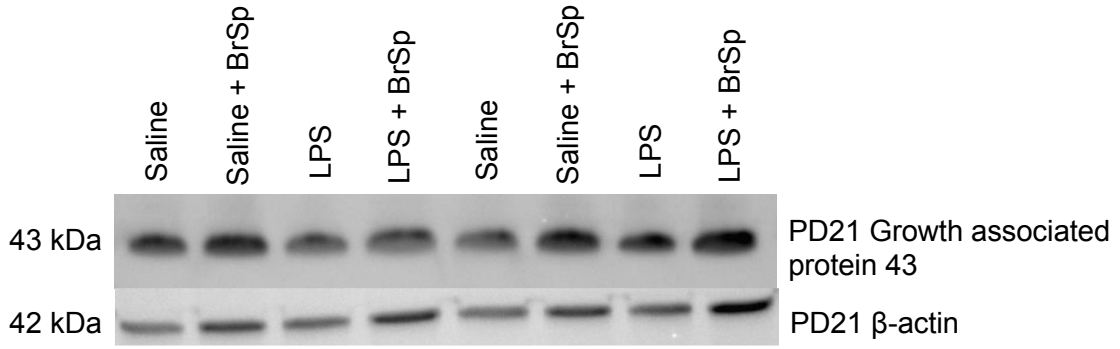

Postnatal Day 7 Growth Associated Protein 43 Expression
Growth Associated Protein 43/ β-actin Immunodensity
0
1
2
3
4
5
6
7
8
(4)
(3)
(3)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

C)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
43 kDa
42 kDa
PD21 Growth associated protein 43
PD21 β-actin


Postnatal Day 21 Growth Associated Protein 43 Expression in the Deep Gray Matter Regions of the Brain
Growth Associated Protein 43/ β-actin Immunodensity
2
1
0
(6)
(6)
(6)
(5)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

D)



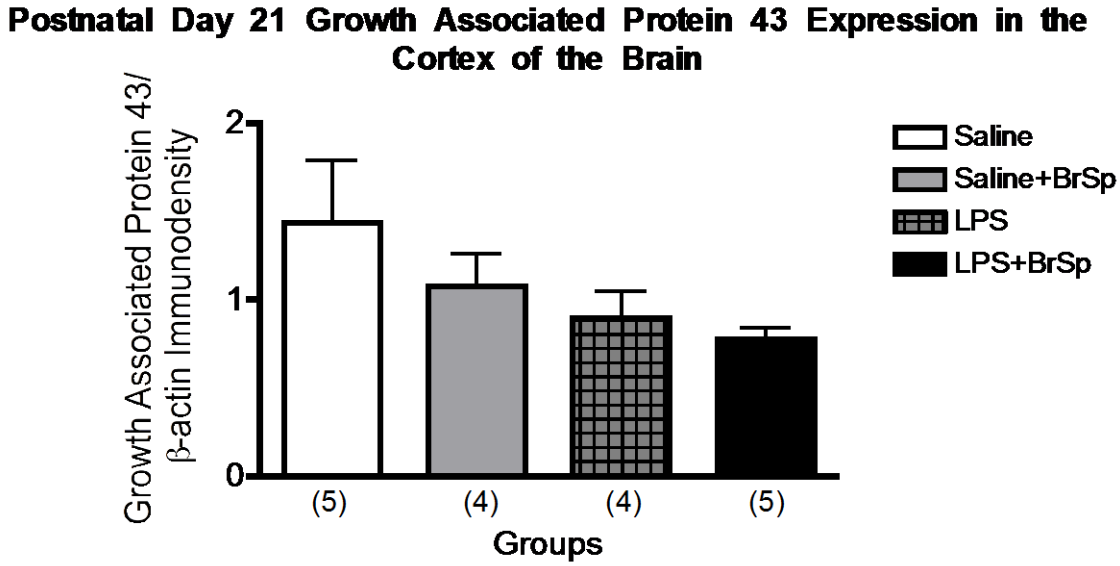
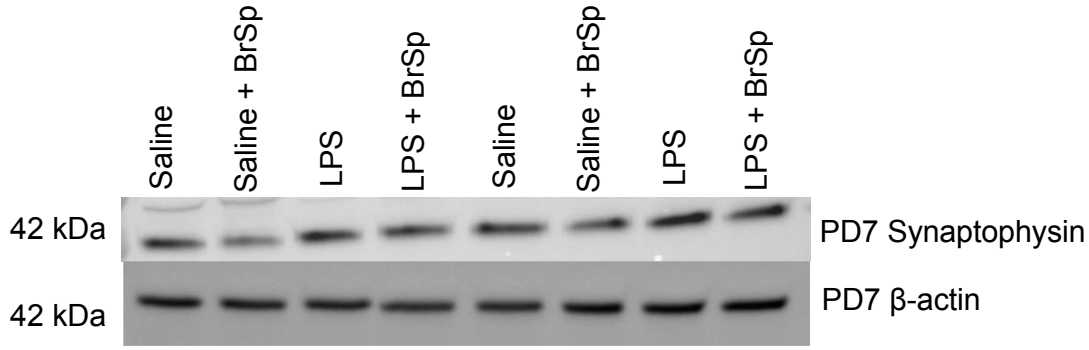
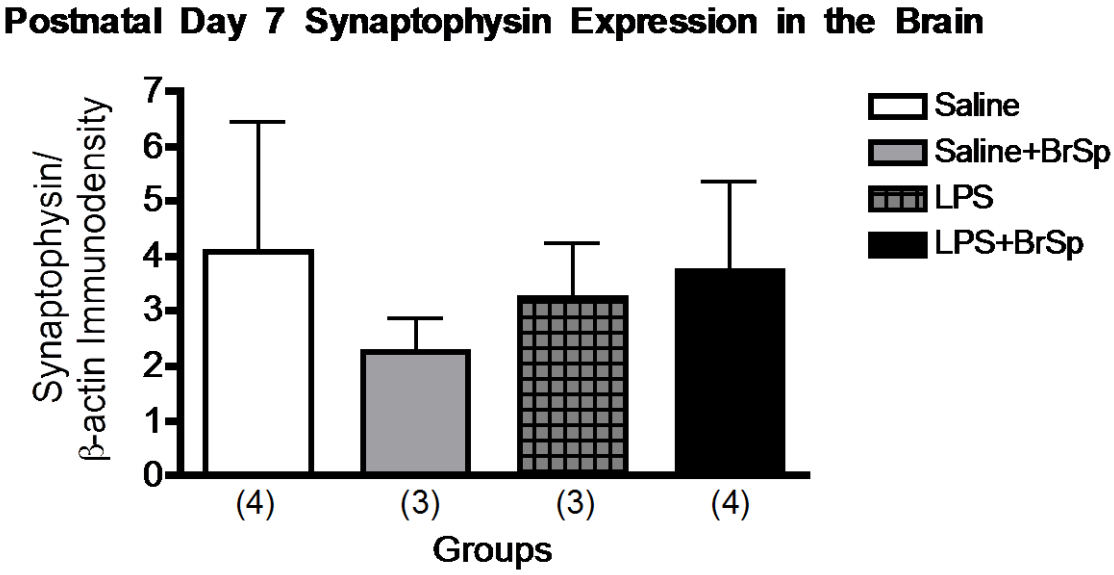


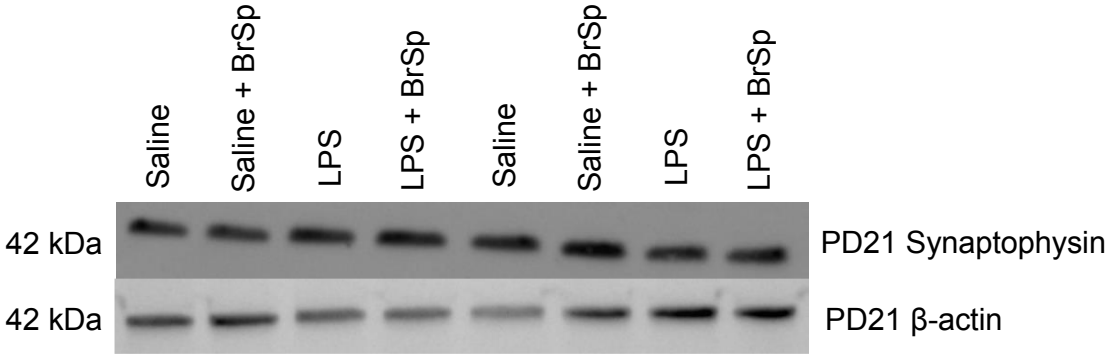
Figure 4.14. Growth associated protein 43 expression in rat brains on PD1, 7, and 21. The expression of growth associated protein 43/β-actin ratio was investigated on PD1 (A), 7 (B), and 21 (C and D). On PD21, two regions were evaluated, the deep gray matter region (C) and cortex (D). No differences were detected. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

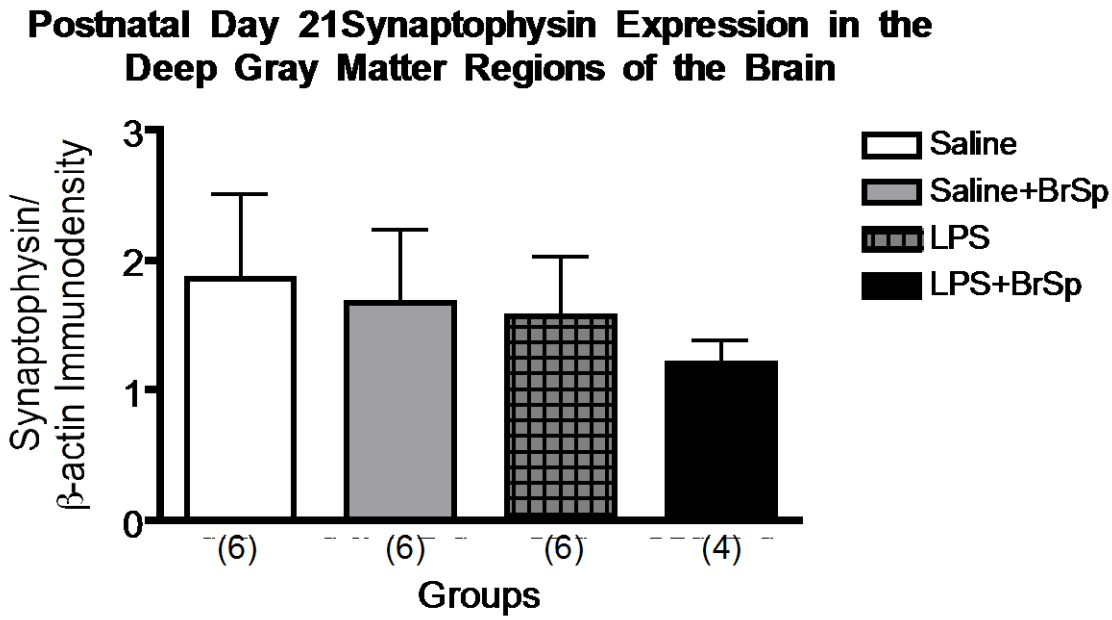
A)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
42 kDa
PD7 Synaptophysin
42 kDa
PD7 β-actin

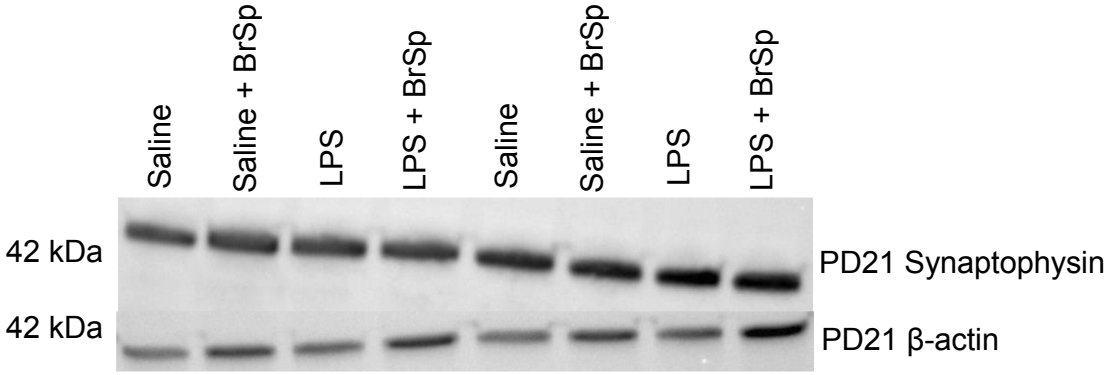

Postnatal Day 7 Synaptophysin Expression in the Brain
Synaptophysin/
β-actin Immunodensity
0
1
2
3
4
5
6
7
(4)
(3)
(3)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

B)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
42 kDa
PD21 Synaptophysin
42 kDa
PD21 β-actin


Postnatal Day 21Synaptophysin Expression in the Deep Gray Matter Regions of the Brain
Synaptophysin/ β-actin Immunodensity
3
2
1
0
(6)
(6)
(6)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

C)



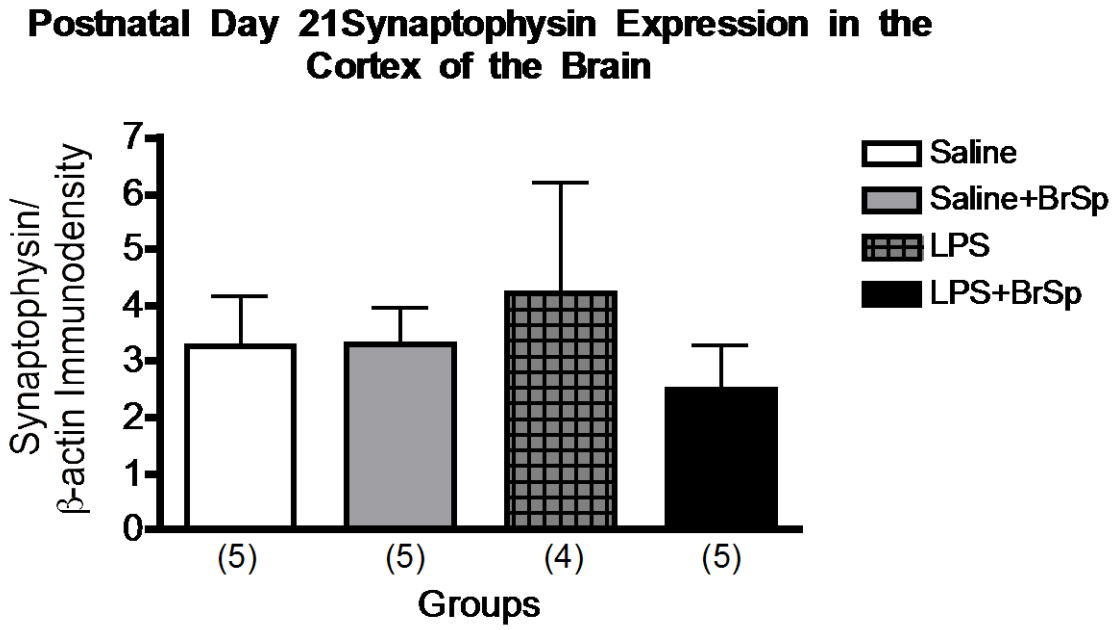
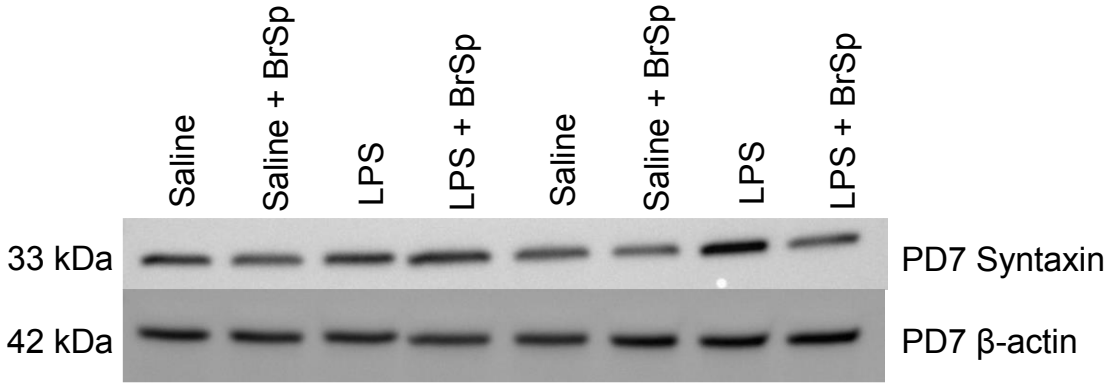
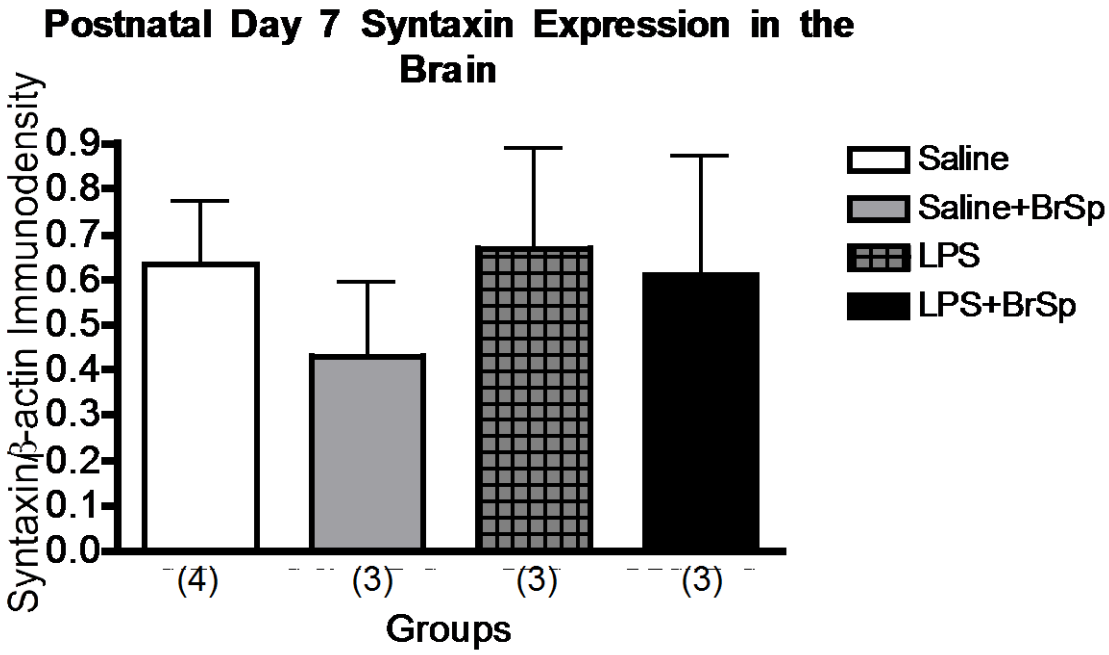


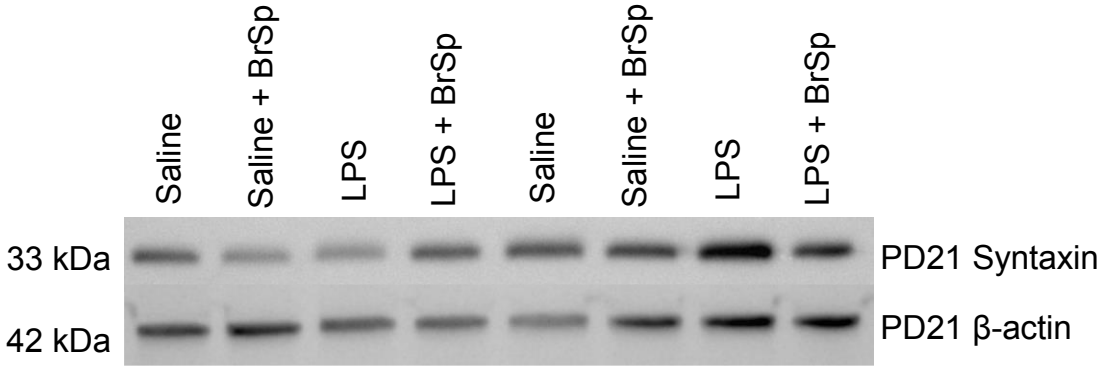
Figure 4.15. Synaptophysin protein expression in the rat brains PD1, 7, and 21. Synaptophysin, a protein found in presynaptic vesicles, is associated with synaptogenesis. The expression of Synaptophysin/ β -actin ratio was analyzed on PD7 (A), PD21 in the deep gray matter (B), and PD21 in the cortex (C). No differences were detected. Data are presented as mean $\pm$ SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

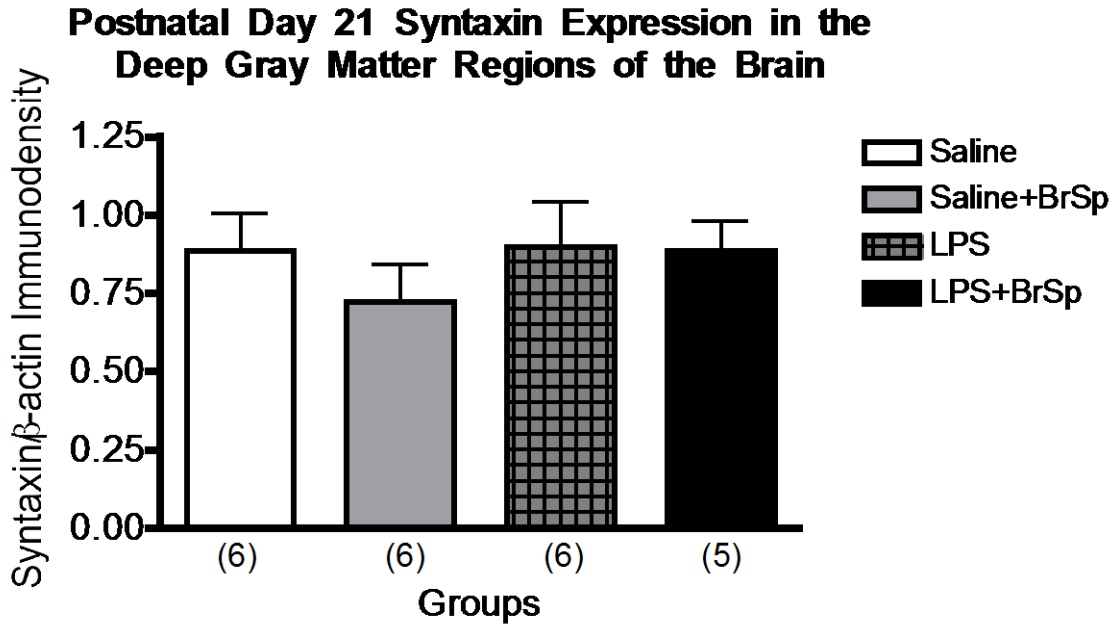
A)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
33 kDa
PD7 Syntaxin
42 kDa
PD7 β-actin

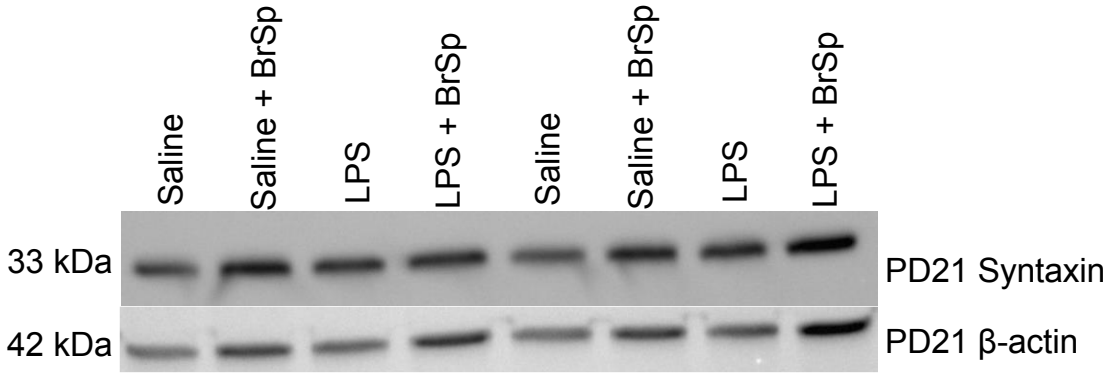

Postnatal Day 7 Syntaxin Expression in the Brain
Syntaxin/β-actin Immunodensity
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(4)
(3)
(3)
(3)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

B)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
33 kDa
42 kDa
PD21 Syntaxin
PD21 β-actin


Postnatal Day 21 Syntaxin Expression in the Deep Gray Matter Regions of the Brain
Syntaxin/β-actin Immunodensity
1.25
1.00
0.75
0.50
0.25
0.00
(6)
(6)
(6)
(5)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

C)



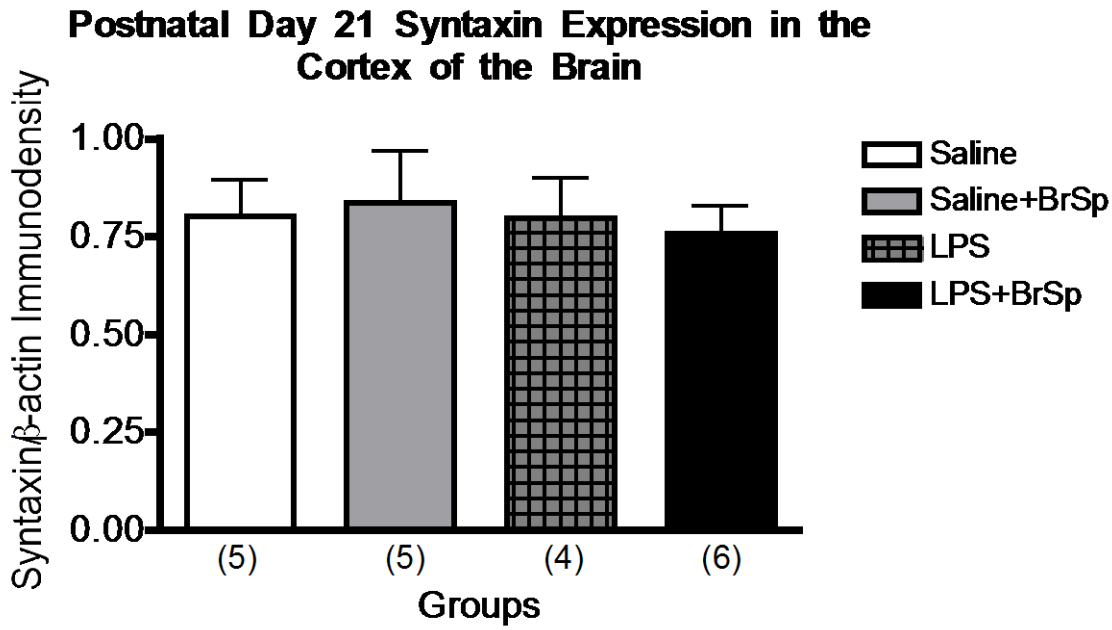
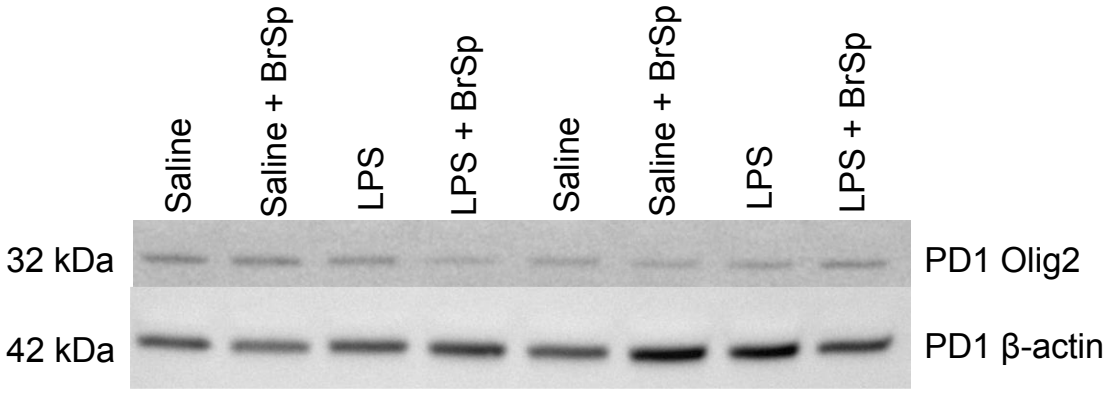
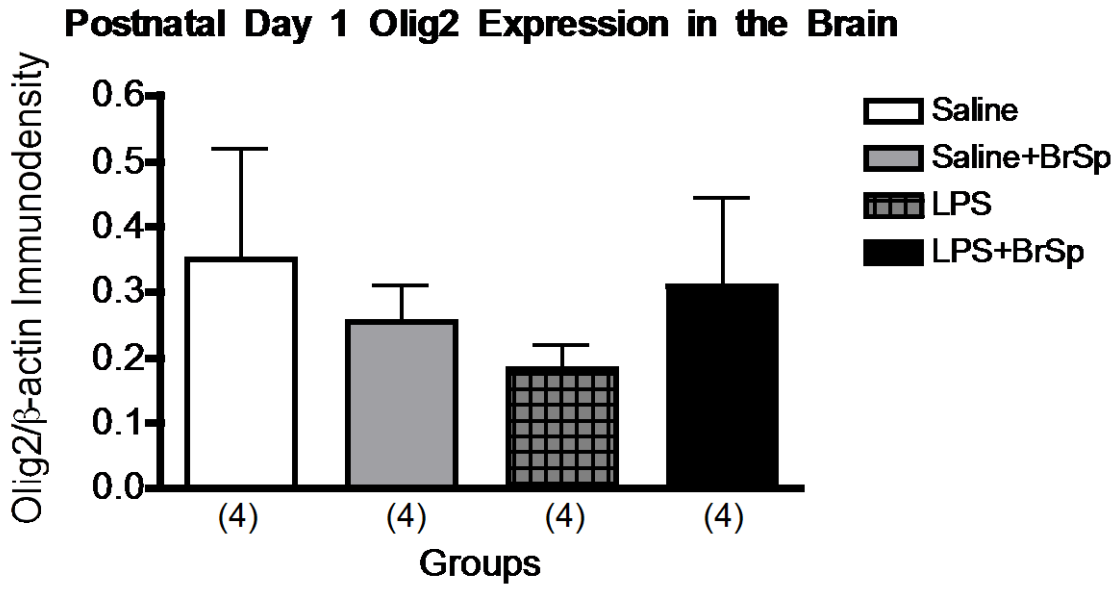


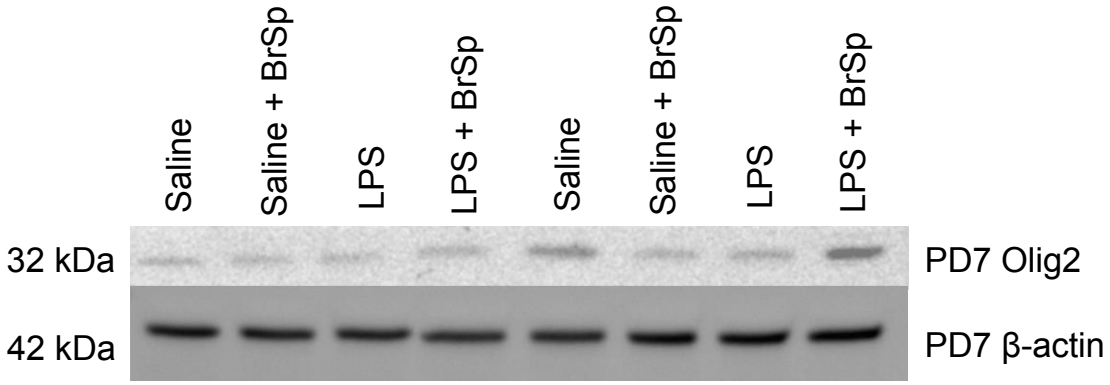
Figure 4.16. Syntaxin protein expression in the rat brains on PD7 and 21. Syntaxin is a protein involved in presynaptic vesicle docking on the membrane that is destined for release into the synaptic cleft. This protein is also a marker of synaptogenesis. The expression of Syntaxin/ β -actin ratio was analyzed on PD7 (A), PD21 in the deep gray matter (B), and PD21 in the cortex (C). No differences were detected. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

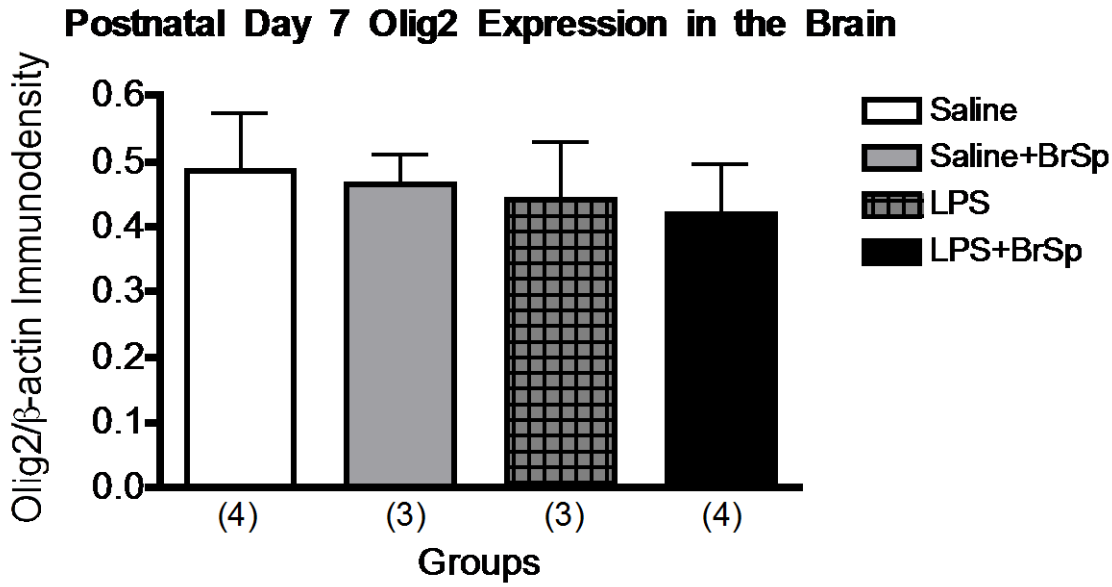
A)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
32 kDa
42 kDa
PD1 Olig2
PD1 β-actin

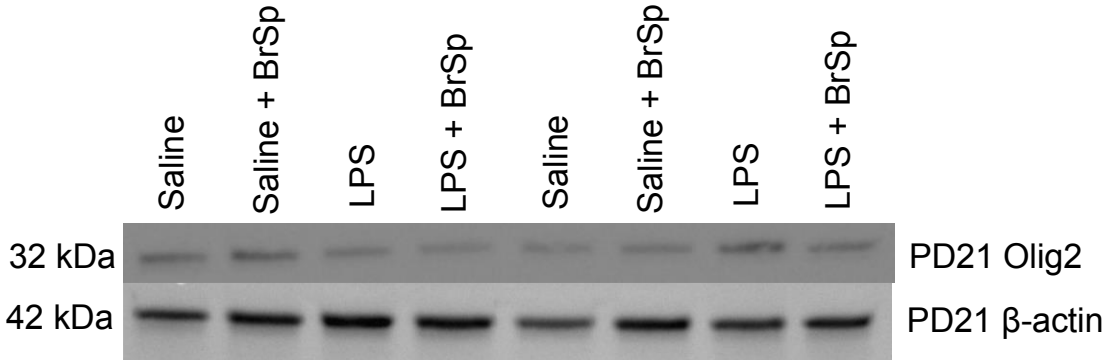

Postnatal Day 1 Olig2 Expression in the Brain
Olig2/β-actin Immunodensity
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(4)
(4)
(4)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

B)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
32 kDa
42 kDa
PD7 Olig2
PD7 β-actin


Postnatal Day 7 Olig2 Expression in the Brain
Olig2/β-actin Immunodensity
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(4)
(3)
(3)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

C)



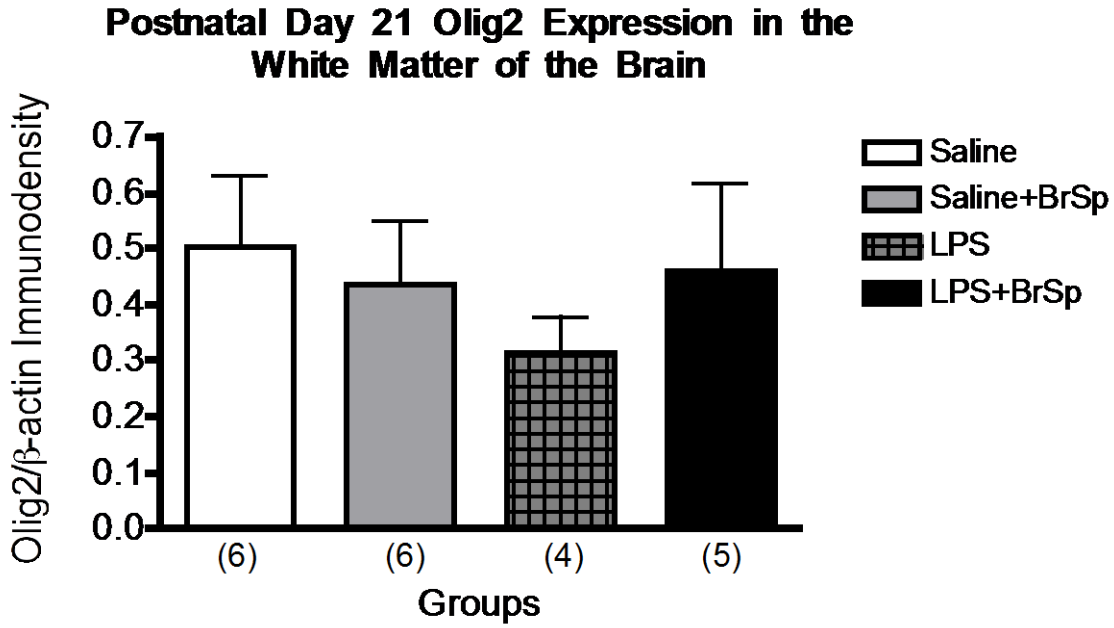
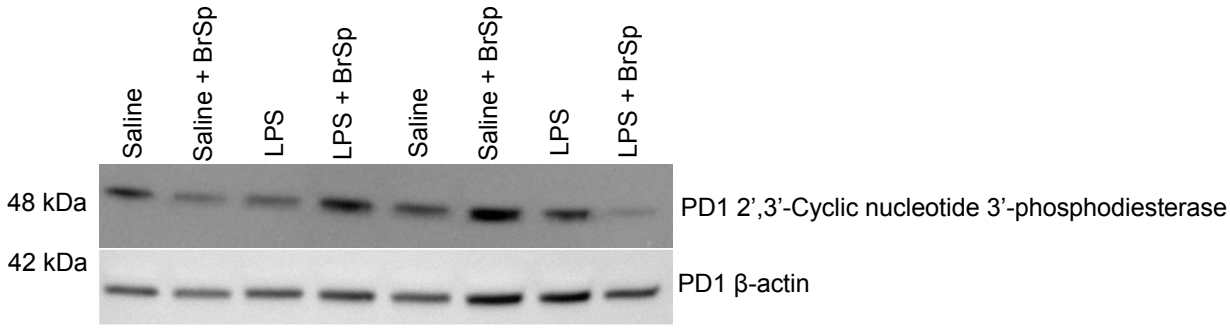
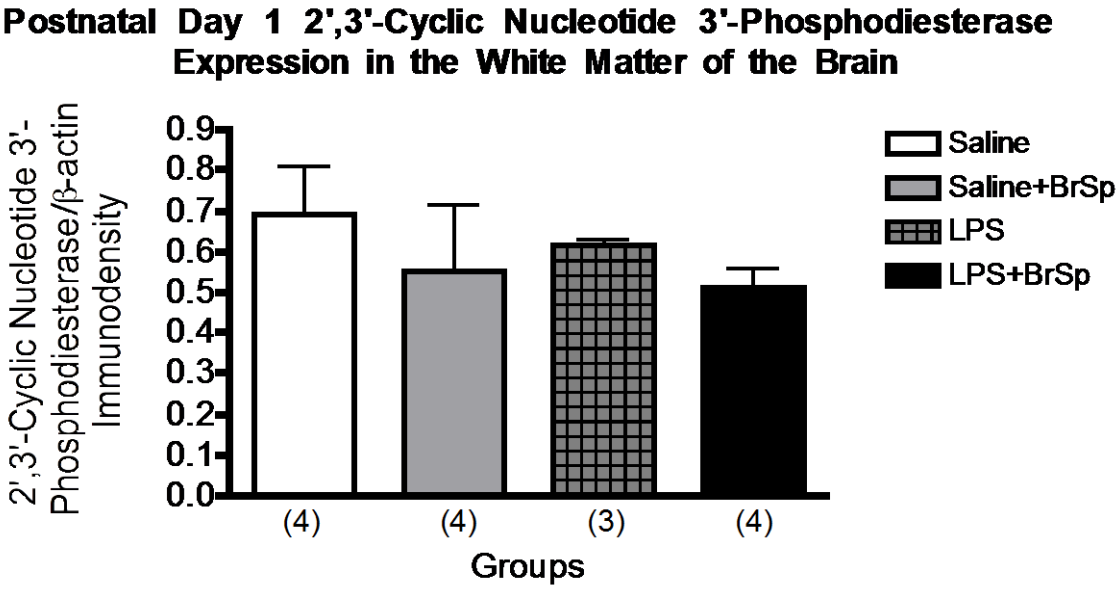


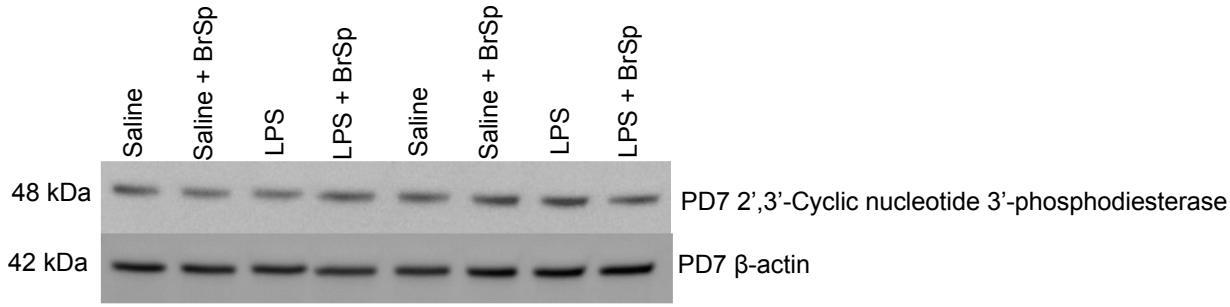
Figure 4.17. Olig2 protein expression in the rat brains on PD1,7, and 21. The expression of the protein Olig2 indicates presence of oligodendrocytes irrespective of maturation stage. The expression of Olig2/ β -actin ratio was analyzed on PD1 (A), PD7 (B), and PD21 in the white matter tract, the corpus callosum (C). No differences were detected. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

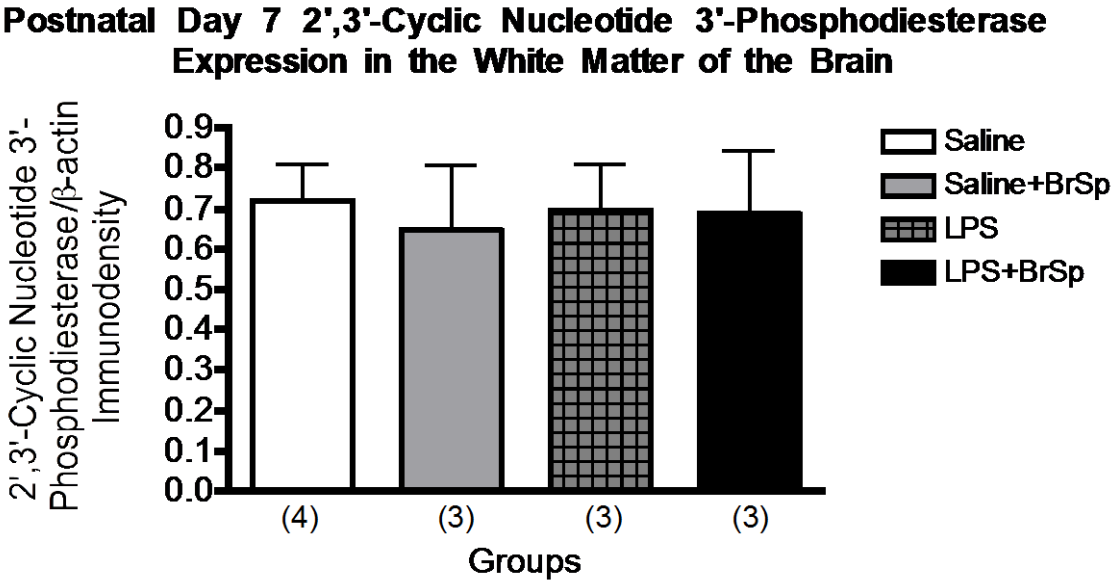
A)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
48 kDa
42 kDa
PD1 2’,3’-Cyclic nucleotide 3’-phosphodiesterase
PD1 β-actin

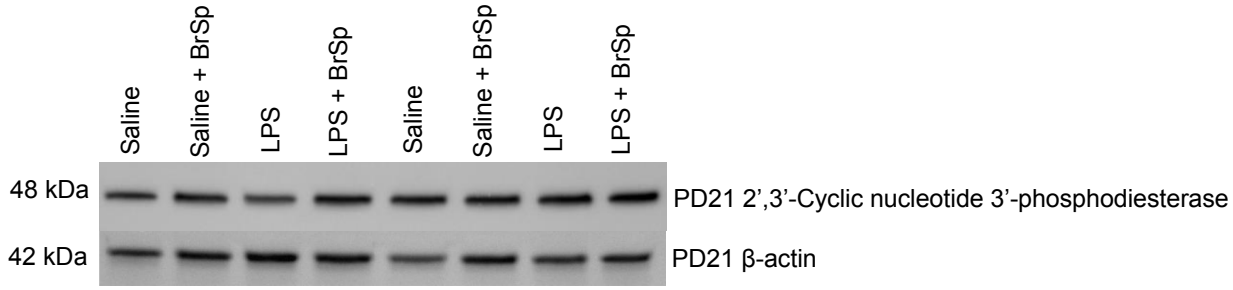

Postnatal Day 1 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase Expression in the White Matter of the Brain
2',3'-Cyclic Nucleotide 3'-Phosphodiesterase/β-actin Immunodensity
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(4)
(4)
(3)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

B)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
48 kDa
42 kDa
PD7 2',3'-Cyclic nucleotide 3'-phosphodiesterase
PD7 β-actin


Postnatal Day 7 2',3'-Cyclic Nucleotide 3'-Phosphodiesterase Expression in the White Matter of the Brain
2',3'-Cyclic Nucleotide 3'-Phosphodiesterase/β-actin Immunodensity
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
(4)
(3)
(3)
(3)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

C)



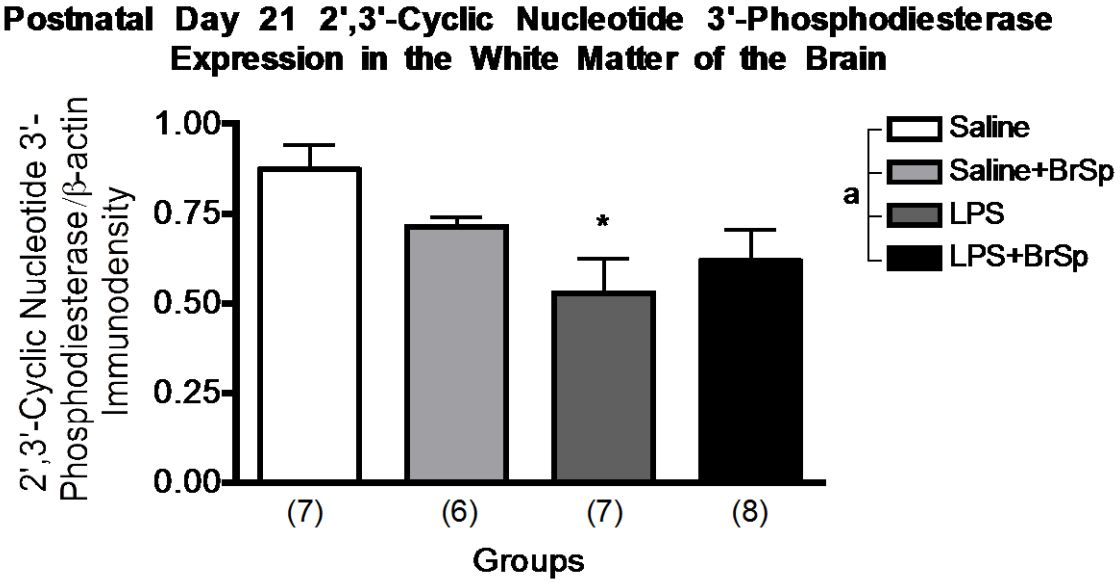
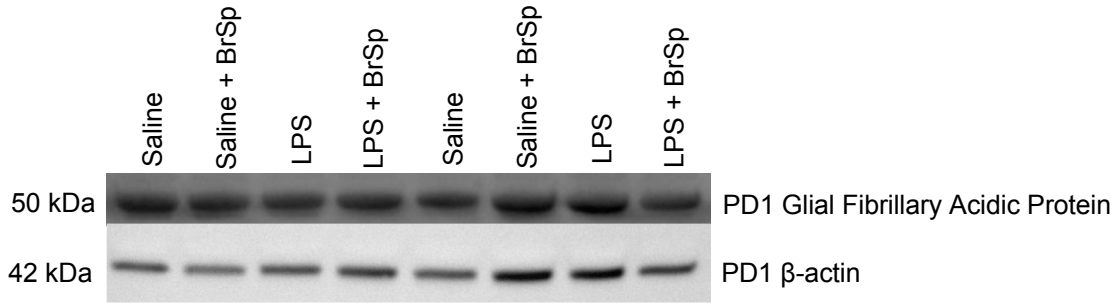
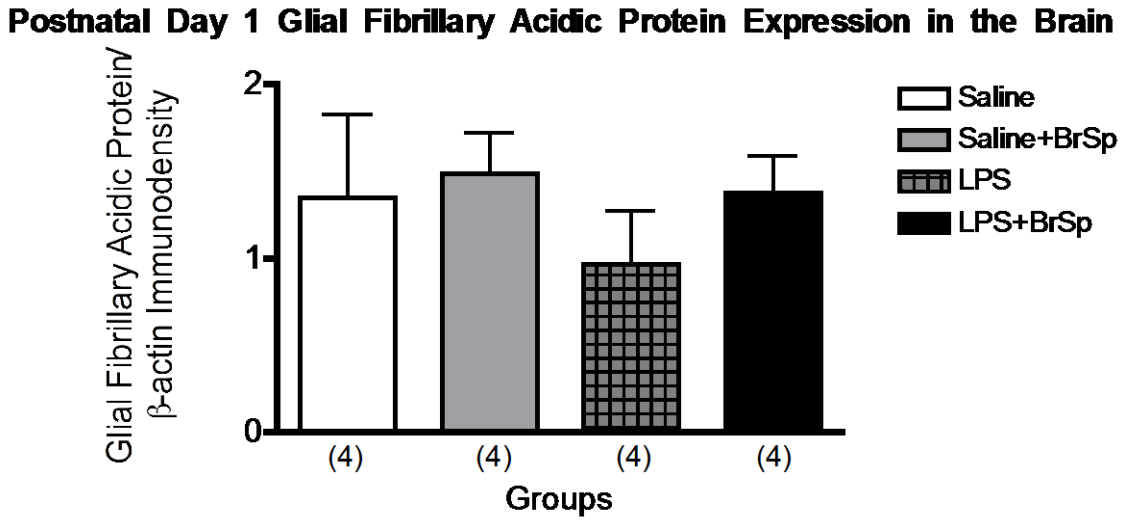


Figure 4.18. 2',3'-Cyclic nucleotide 3'-phosphodiesterase protein expression in the rat brains on PD1,7, and 21. The expression of the protein 2',3'-Cyclic nucleotide 3'-phosphodiesterase is a marker of differentiating oligodendrocytes. The expression of 2',3'-Cyclic nucleotide 3'-phosphodiesterase/ β -actin ratio was analyzed on PD1 (A), PD7 (B), and PD21 in the white matter tract, the corpus callosum (C). On PD21 in the white matter tract, the corpus callosum, a significant main effect of Treatment was detected. LPS pups had decreased expression of 2',3'-cyclic-nucleotide 3'-phosphodiesterase compared to Saline pups. Data are presented as mean $\pm$ SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group. a =

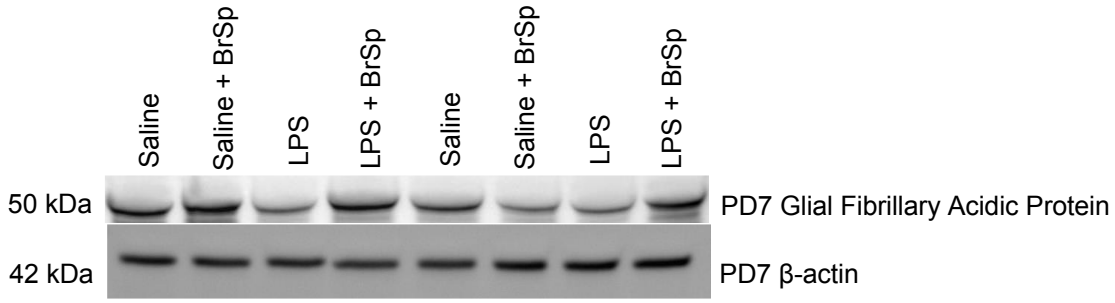
significant main effect of Treatment. * = significant difference between Treatment and control (i.e. LPS versus Saline or LPS + BrSp vs Saline + BrSp).

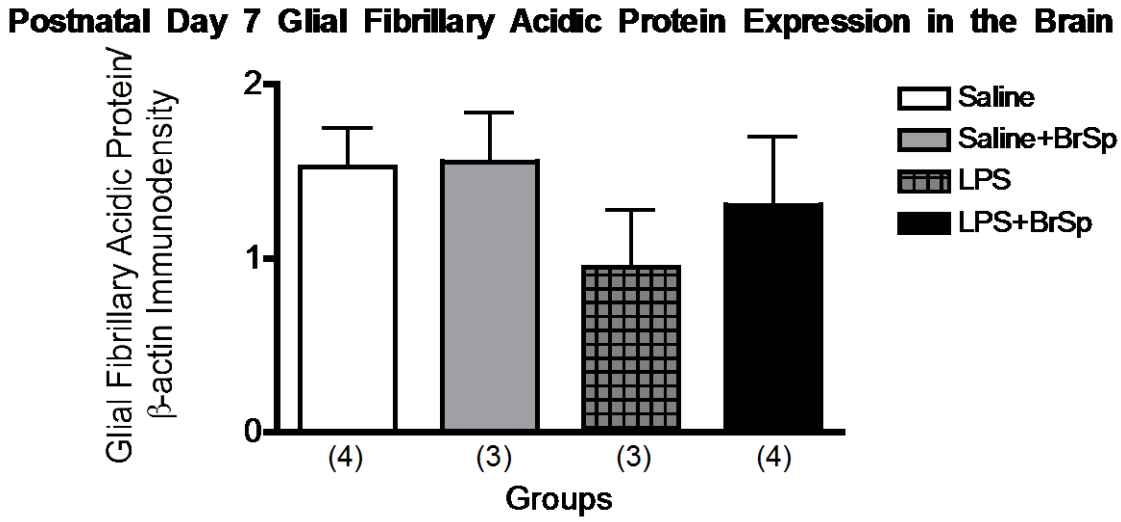
A)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
50 kDa
PD1 Glial Fibrillary Acidic Protein
42 kDa
PD1 β-actin

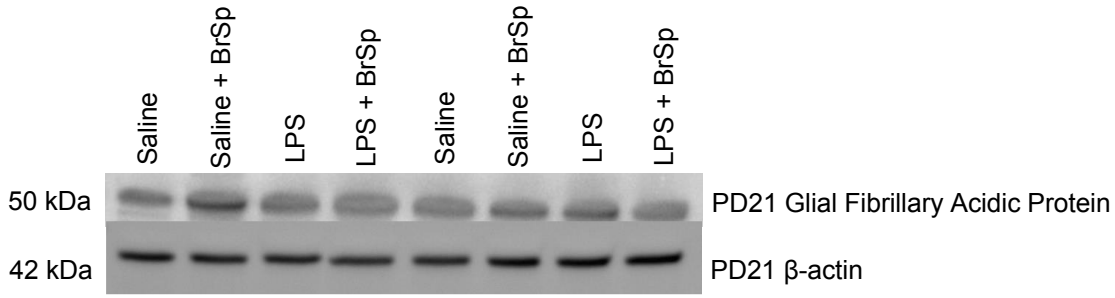

Postnatal Day 1 Glial Fibrillary Acidic Protein Expression in the Brain
Glial Fibrillary Acidic Protein/ β-actin Immunodensity
2
1
0
(4)
(4)
(4)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

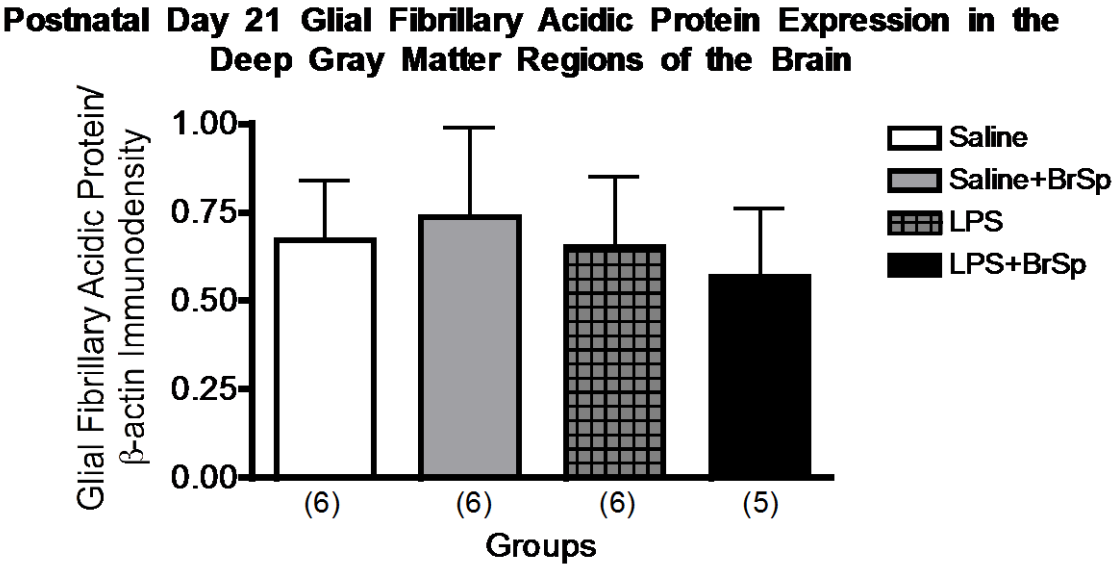
B)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
50 kDa
42 kDa
PD7 Glial Fibrillary Acidic Protein
PD7 β-actin

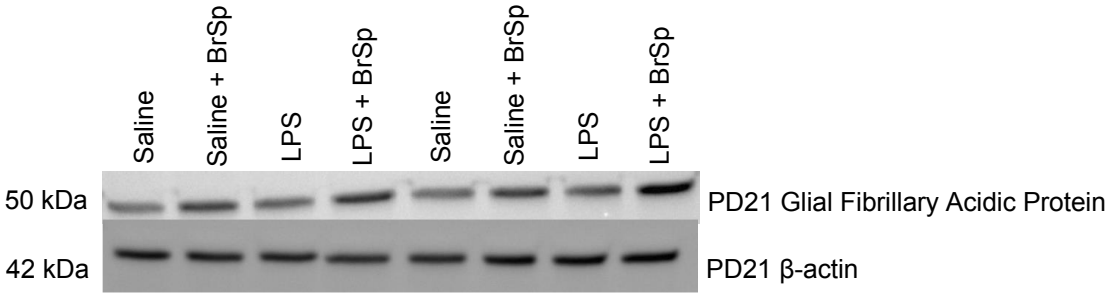

Postnatal Day 7 Glial Fibrillary Acidic Protein Expression in the Brain
Glial Fibrillary Acidic Protein/ β-actin Immunodensity
2
1
0
(4)
(3)
(3)
(4)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

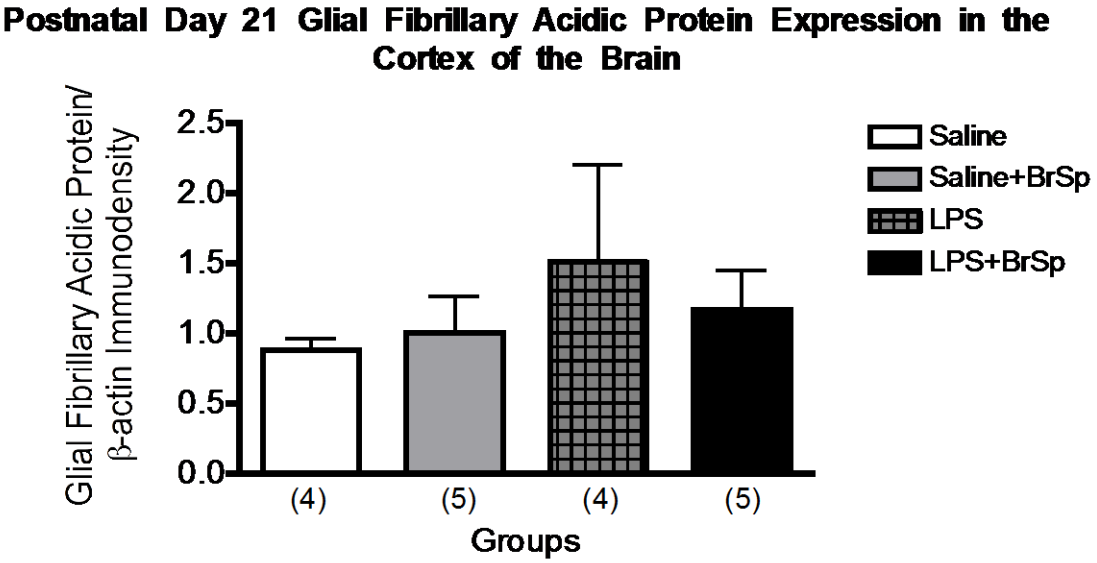
C)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
50 kDa
42 kDa
PD21 Glial Fibrillary Acidic Protein
PD21 β-actin

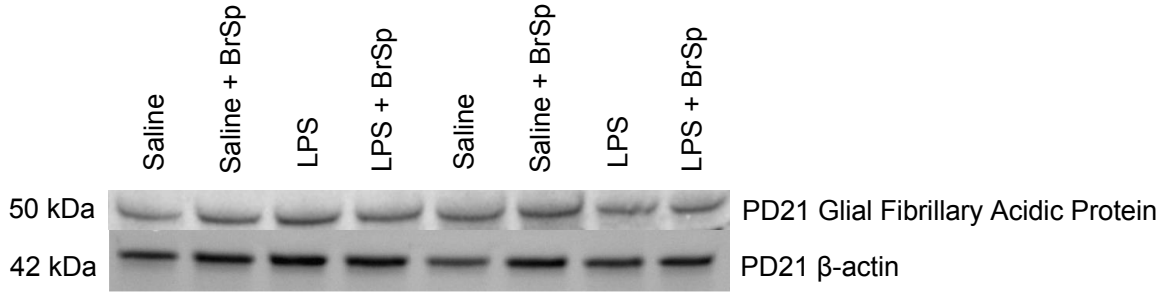

Postnatal Day 21 Glial Fibrillary Acidic Protein Expression in the Deep Gray Matter Regions of the Brain
Glial Fibrillary Acidic Protein/ β-actin Immunodensity
1.00
0.75
0.50
0.25
0.00
(6)
(6)
(6)
(5)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

D)


Saline
Saline + BrSp
LPS
LPS + BrSp
Saline
Saline + BrSp
LPS
LPS + BrSp
50 kDa
42 kDa
PD21 Glial Fibrillary Acidic Protein
PD21 β-actin


Postnatal Day 21 Glial Fibrillary Acidic Protein Expression in the Cortex of the Brain
Glial Fibrillary Acidic Protein/ β-actin Immunodensity
2.5
2.0
1.5
1.0
0.5
0.0
(4)
(5)
(4)
(5)
Groups
Saline
Saline+BrSp
LPS
LPS+BrSp

E)



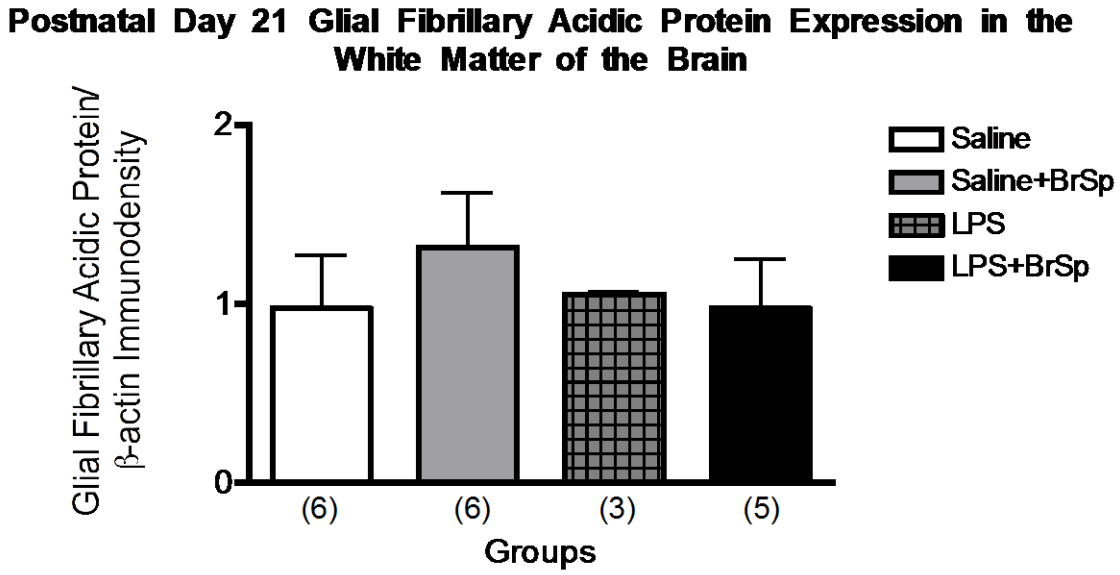


Figure 4.19. Expression of glial fibrillary acidic protein in rat brains on PD1,7, and 21. Glial fibrillary acidic protein was measured in offspring brains to detect astrogliosis. The expression of glial fibrillary acid protein/ β -actin ratio was analyzed on PD1 (A), PD7 (B), and PD21 in the deep gray matter regions (C), cortex (D), and the white matter tract (E). No differences were detected. Data are presented as mean $\pm$ SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

4.3. OBJECTIVE 3

Chorioamnionitis results from maternal infection and inflammation, thus it is not surprising that maternal physiological responses would be altered. During pregnancy, maternal immunology is altered so that the fetus is not rejected, and proper development of the placenta is essential to maintain fetal viability and growth. The uterine arteries feed into the placenta, and indirectly the fetus. During chorioamnionitis, the immunological response may be altered, causing uterine endothelial dysfunction via proinflammatory mediators. This may partially explain the intrauterine growth restricted offspring from LPS dams. The next objective was to identify mechanistic changes to maternal uterine vasculature following *in* utero inflammation. This objective was aimed to identify pathological mechanisms that may be a potential therapeutic target to prevent fetal growth restriction.

4.3.1. Uteroplacental and Umbilical-Placental Blood Flow

The pulsatility and resistance indices of both the uterine and umbilical arteries were assessed to determine the effects of LPS and BrSp on blood flow on E21. Comparisons between Saline and LPS uterine arteries did not reveal any differences in pulsatility index (1.2 ± 0.1 and 1.1 ± 0.05, respectively) (Figure 4.20A) or resistance index (0.7 ± 0.05 and 0.7 ± 0.02, respectively) (Figure 4.20B), suggesting that uterine blood flow is not impeded. Since no differences were detected, BrSp groups were not pursued. Similarly, data comparing Saline and LPS umbilical arteries did not reveal differences on pulsatility index (1.8 ± 0.05 and 1.8 ± 0.04, respectively) (Figure 4.20C) and resistance index (1.0 ± 0.01 and 1.0 ± 0.01, respectively)

(Figure 4.20D). This suggests that fetal blood flow is also not disrupted or affected by LPS exposure, therefore BrSp groups were not investigated.

4.3.2. Uterine Artery Vascular Response

We assessed uterine artery vascular function *ex vivo* on E22. Uterine arteries were mounted in wire myograph baths and each artery was exposed to the following drugs: no drugs (control), 1400W (inducible nitric oxide synthase inhibitor), pegSOD (O_2^- scavenger), and L-NAME (pan-nitric oxide synthase inhibitor). A cumulative concentration response curve was performed following exposure to the α_1-adrenoreceptor agonist phenylephrine (vasoconstriction) and the muscarinic receptor agonist methacholine (endothelial-dependent vasodilation). The cumulative concentration response curve was used to calculate the pEC_{50} dose required to elicit a 50% response (potency) of maximal contraction or dilation, and the E_{max}, the maximal constriction or dilation induced during the dose response curve (signaling pathway efficacy).

4.3.3. Phenylephrine Cumulative Concentration Response Curves

The phenylephrine cumulative concentration response curve was calculated following incubation of arteries with the abovementioned inhibitors or the free radical scavenger superoxide dismutase (Figure 4.21A). *In vivo* inflammation did not alter the pEC_{50} (Figure 4.21B) between Saline (6.1 ± 0.02) and LPS (6.0 ± 0.2). This suggests that LPS and Saline did not differ in receptor sensitivity to phenylephrine. LPS arteries (9.4 ± 2.4 mN/mm) had a reduced

E_{max} compared to Saline arteries (12.8 ± 1.8 mN/mm), however the hyporeactivity was not significant (Figure 4.21C).

The cumulative concentration response curve resulting from incubation of arteries with 1400W (Figure 4.22A) did not elicit differences to the pEC_{50} (Figure 4.22B) between the Saline (6.1 ± 0.07) and LPS (6.3 ± 0.2) groups. Furthermore, E_{max} (Figure 4.22C) values in the Saline + 1400W (11.3 ± 0.9 mN/mm) and LPS + 1400W (9.3 ± 2.1 mN/mm) did not differ.

Following incubation with pegSOD (Figure 4.23A), no differences in pEC_{50} (Figure 4.23B) values between Saline (6.1 ± 0.02) and LPS (6.1 ± 0.2) groups were detected. Furthermore, no changes were observed upon analyses of E_{max} (Figure 4.23C) in the Saline + pegSOD (11.0 ± 0.4 mN/mm) and LPS + pegSOD (10.9 ± 0.8 mN/mm) groups.

No differences in the phenylephrine cumulative concentration response curve were observed following incubation with L-NAME (Figure 4.24A). The pEC_{50} between Saline + L-NAME (6.2 ± 0.05) and LPS + L-NAME (6.3 ± 0.4) also did not differ (Figure 4.24B). Similarly, Saline + L-NAME (11.8 ± 2.0 mN/mm) and LPS + L-NAME (9.8 ± 2.3 mN/mm) E_{max} values did not differ (Figure 4.24C).

4.3.4. Methacholine Cumulative Concentration Response Curves

Uterine arteries were incubated with their respective drugs followed by a preconstriction (EC_{80}) dose to 80% of their maximal constriction determined from the prior phenylephrine

cumulative concentration response curve. The relaxation curve was performed using methacholine, an endothelial dependent vasodilator. No differences were identified between Saline and LPS arteries on their vasodilatory response (Figure 4.25A). In addition, no differences were observed in the pEC_{50} between Saline (8.6 ± 0.6) and LPS (7.7 ± 0.5) groups (Figure 4.25B). No differences on E_{max} values between Saline (97.6 ± 0.8%) and LPS (83.1 ± 13.2%) were identified (Figure 4.25C).

Similarly, incubation with 1400W did not result in any differences following analyses of the cumulative concentration response curve (Figure 4.26A). Incubation with 1400W did not alter pEC_{50} values in either Saline (7.2 ± 0.7) or LPS (8.5 ± 1.1) groups. (Figure 4.26B). Similarly, E_{max} values did not differ between Saline + 1400W (95.5 ± 2.3%) and LPS + 1400W (94.8 ± 3.8%) groups (Figure 4.26C).

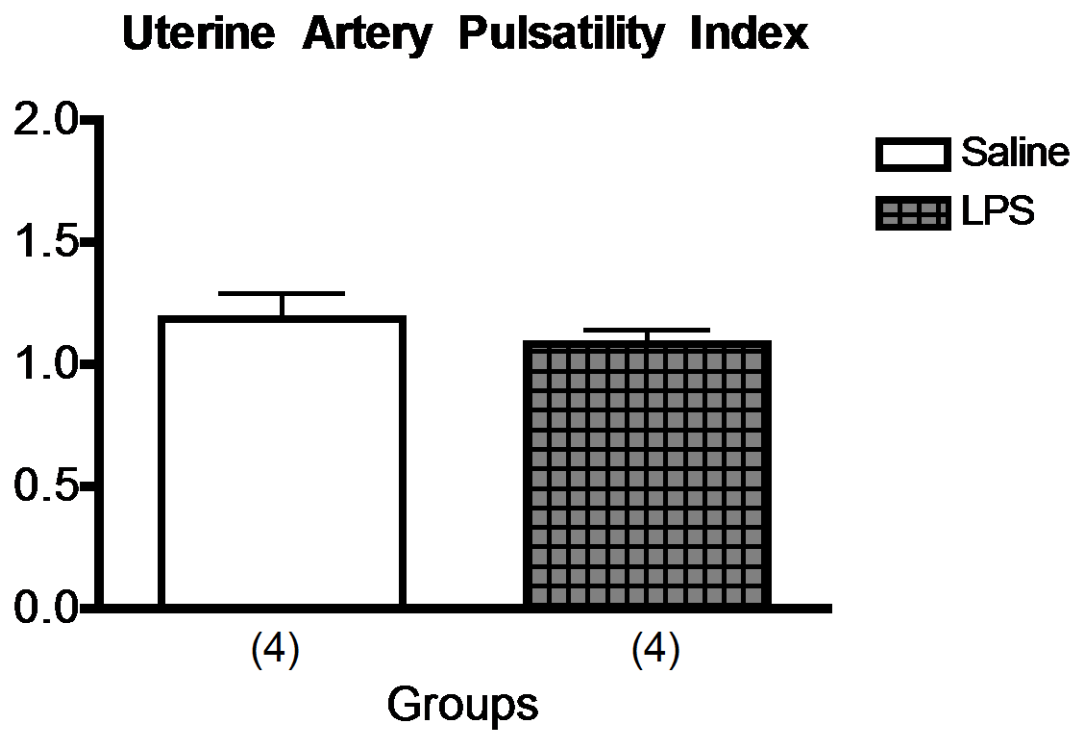
Arteries were incubated with pegSOD followed by cumulative doses of methacholine to induce relaxation (Figure 4.27A). The pEC_{50} values obtained for Saline + pegSOD (8.5 ± 0.4) did not differ from those obtained for LPS + pegSOD (8.3 ± 1.0) (Figure 4.27B). E_{max} values for Saline (99.0 ± 1.3%) and LPS (95.1 ± 1.8%) were not statistically significant from one another (Figure 4.27C).

Following incubation with L-NAME (Figure 4.28A), pEC_{50} did not differ between Saline arteries (7.6 ± 0.7) and LPS arteries (6.2 ± 0.8) (Figure 4.28B). Maximal vasodilation between Saline + L-NAME arteries (51.2 ± 45.0 %) and LPS + L-NAME arteries (64.2 ± 27.9%) (Figure 4.28C).

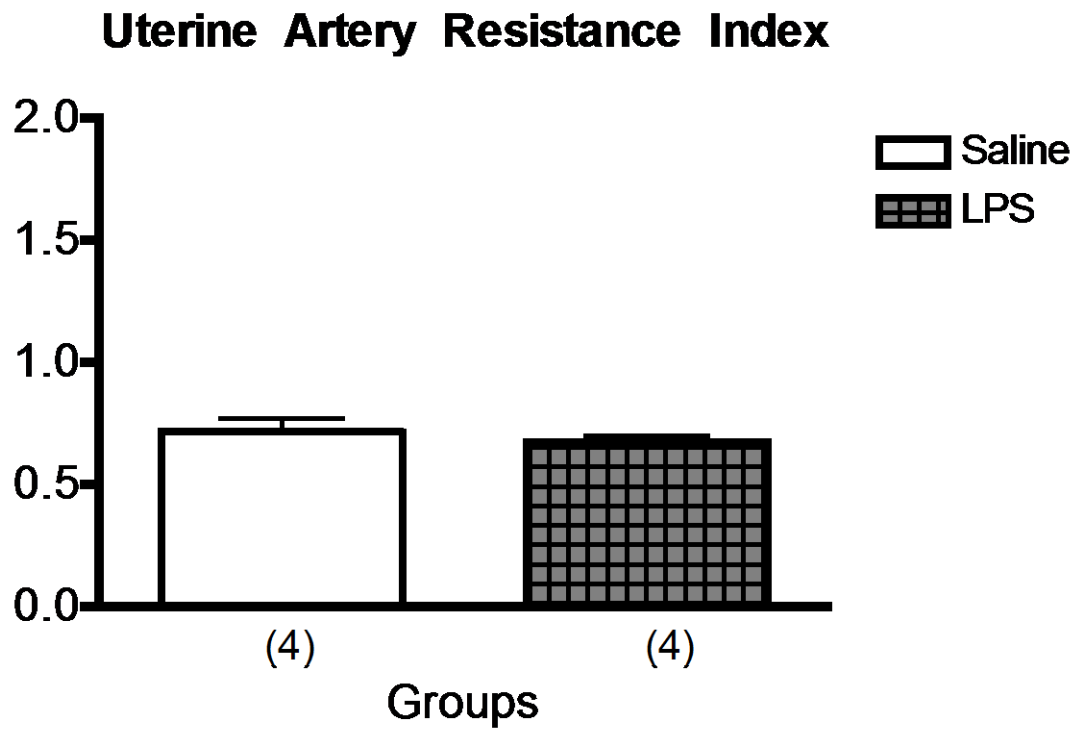
4.3.5. Summary

On E22, we did not detect differences in pulsatility or resistance index between Saline and LPS dams, suggesting that blood flow via the uterine and umbilical arteries were not altered. No differences were detected in uterine artery contractile responses to the vasopressor agent phenylephrine between Saline and LPS groups. Furthermore, incubation with 1400W, pegSOD, and L-NAME did not elicit any differences between the groups. No differences were observed in endothelial dependent relaxation in the uterine arteries following exposure to methacholine. Incubation with 1400W, pegSOD, and L-NAME also did not elicit any differences in vascular relaxation responses between Saline and LPS. Overall, this inflammatory model appears not to alter blood flow and uterine artery function.

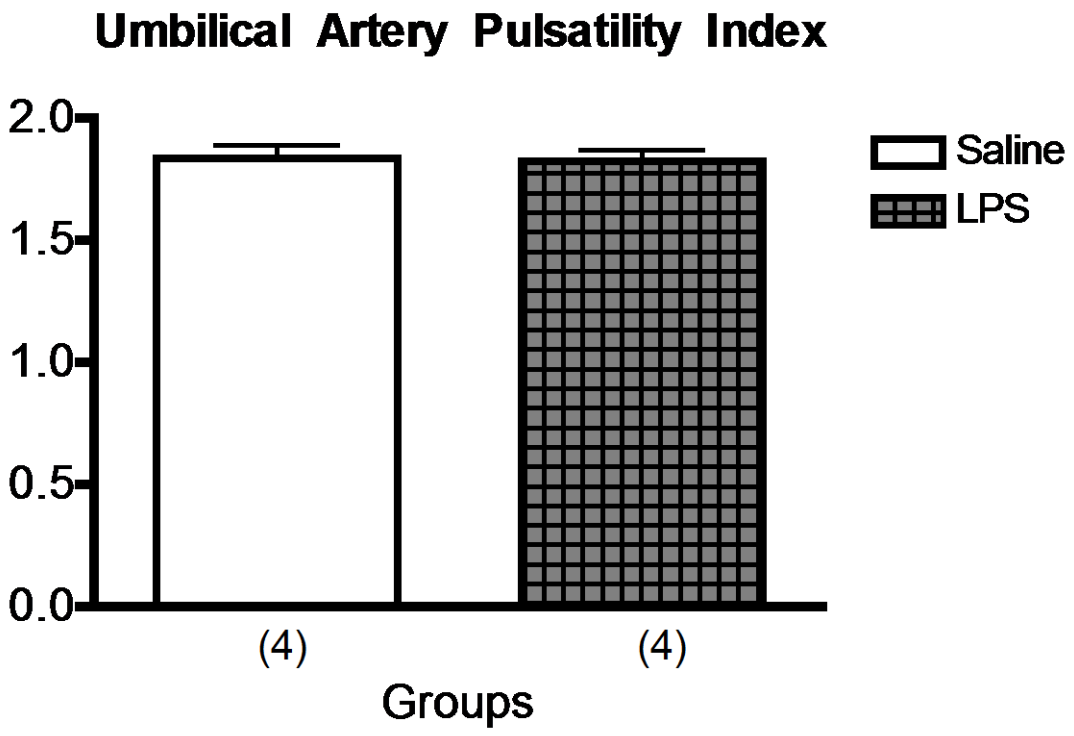
A)


Uterine Artery Pulsatility Index
2.0
1.5
1.0
0.5
0.0
Saline
LPS
(4)
(4)
Groups

B)


Uterine Artery Resistance Index
2.0
1.5
1.0
0.5
0.0
Saline
LPS
(4)
(4)
Groups

C)


Umbilical Artery Pulsatility Index
2.0
1.5
1.0
0.5
0.0
Saline
LPS
(4)
(4)
Groups

D)

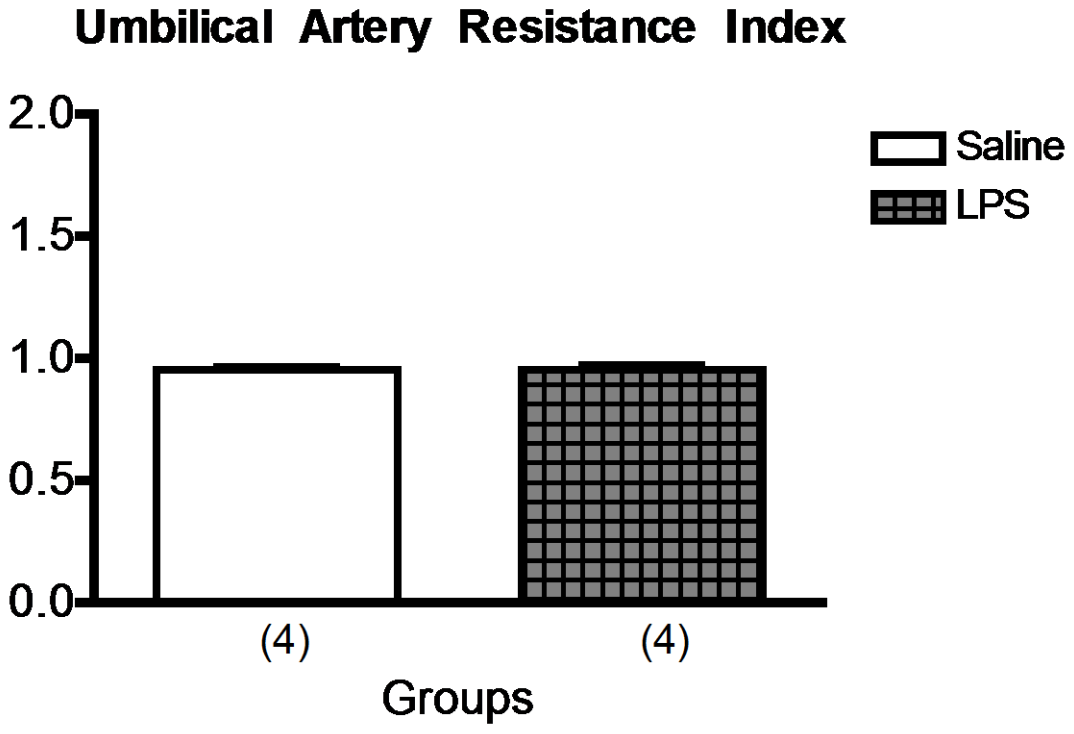
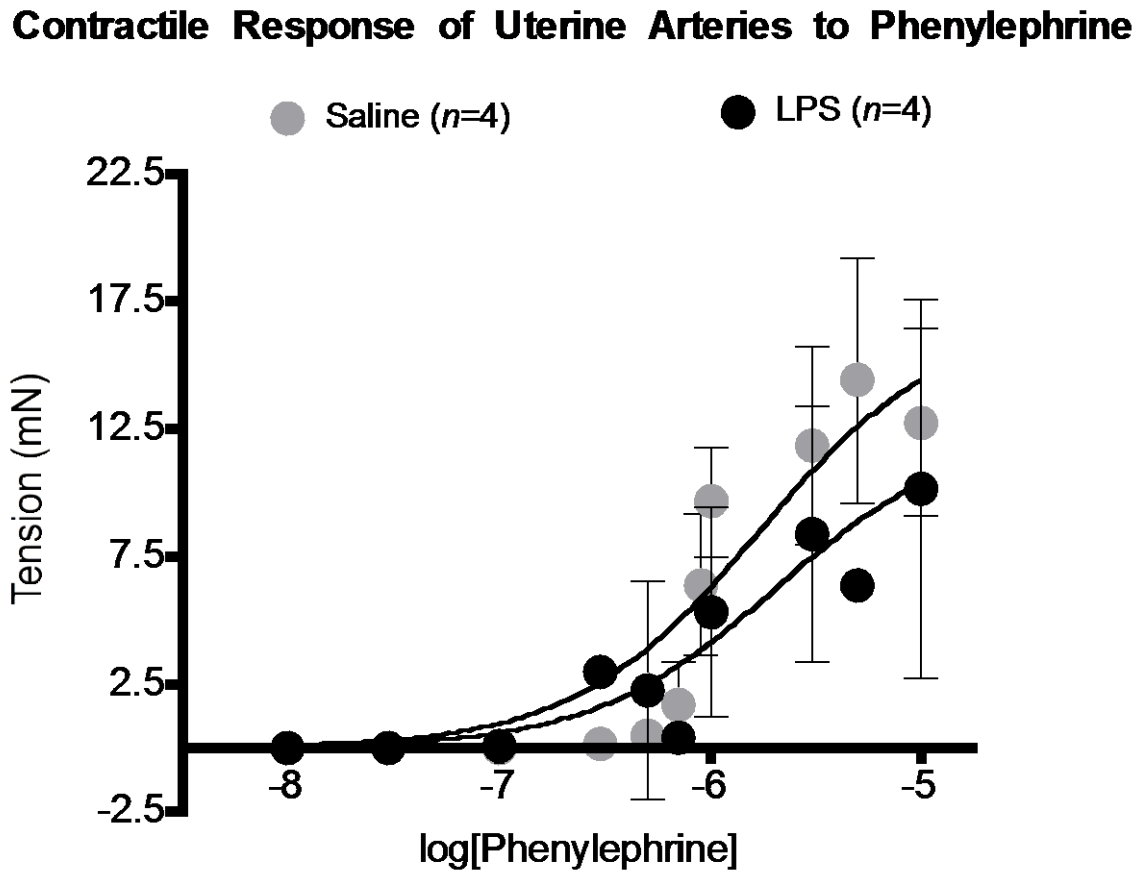
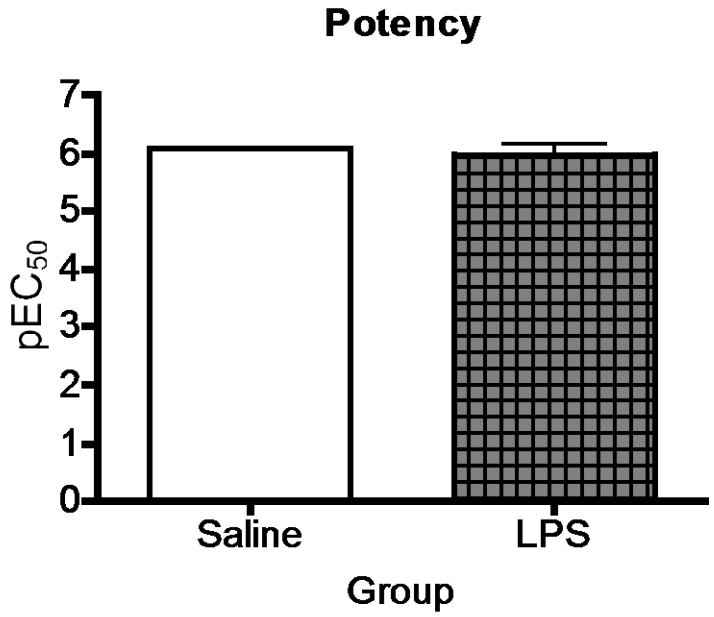

Umbilical Artery Resistance Index
2.0
1.5
1.0
0.5
0.0
Saline
LPS
(4)
(4)
Groups

Figure 4.20. E21 uterine and umbilical pulsatility and resistance index measurements. Uterine and umbilical arteries were assessed for impedance to blood flow. On E21, uterine arteries were assessed for pulsatility (A) and resistance (B) indices. Similarly, the umbilical arteries were analyzed for pulsatility (C) and resistance (D) indices. No differences were detected between Saline and LPS dams. Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

A)


Contractile Response of Uterine Arteries to Phenylephrine
Saline (n=4)
LPS (n=4)
Tension (mN)
22.5
17.5
12.5
7.5
2.5
-2.5
-8
-7
-6
-5
log[Phenylephrine]

B)



C)

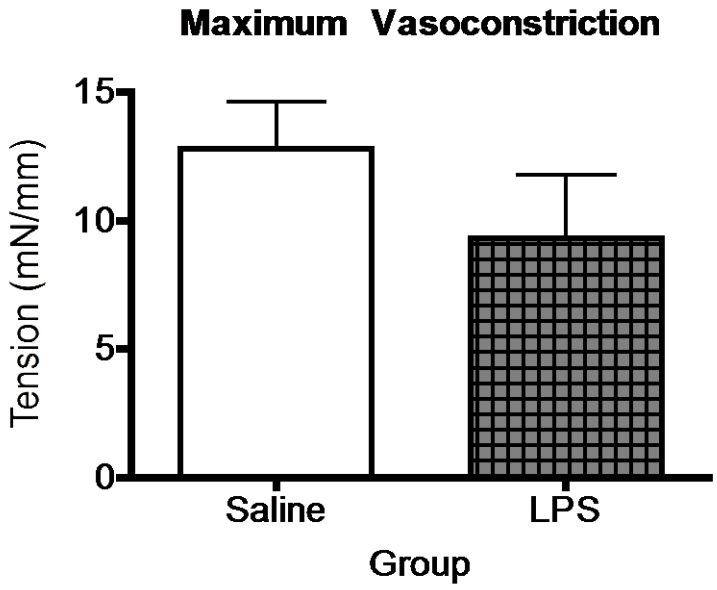
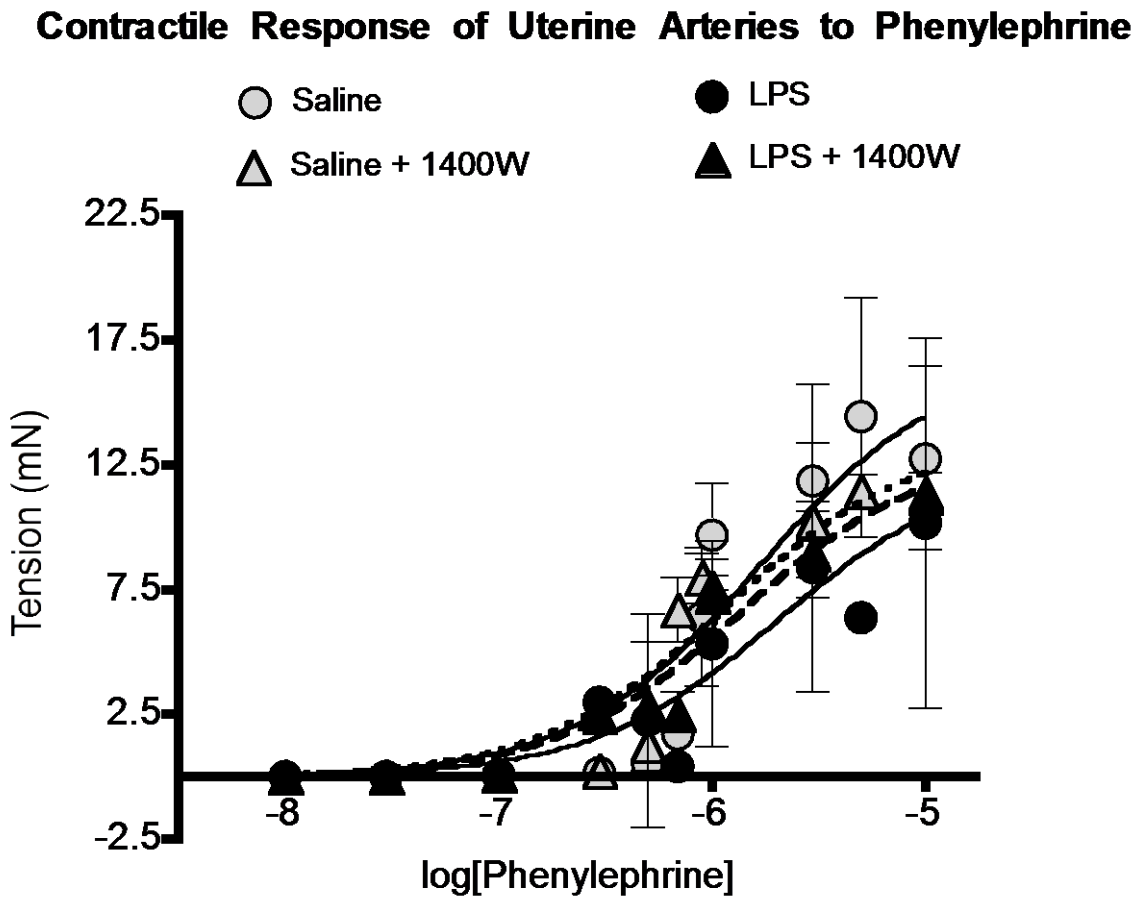
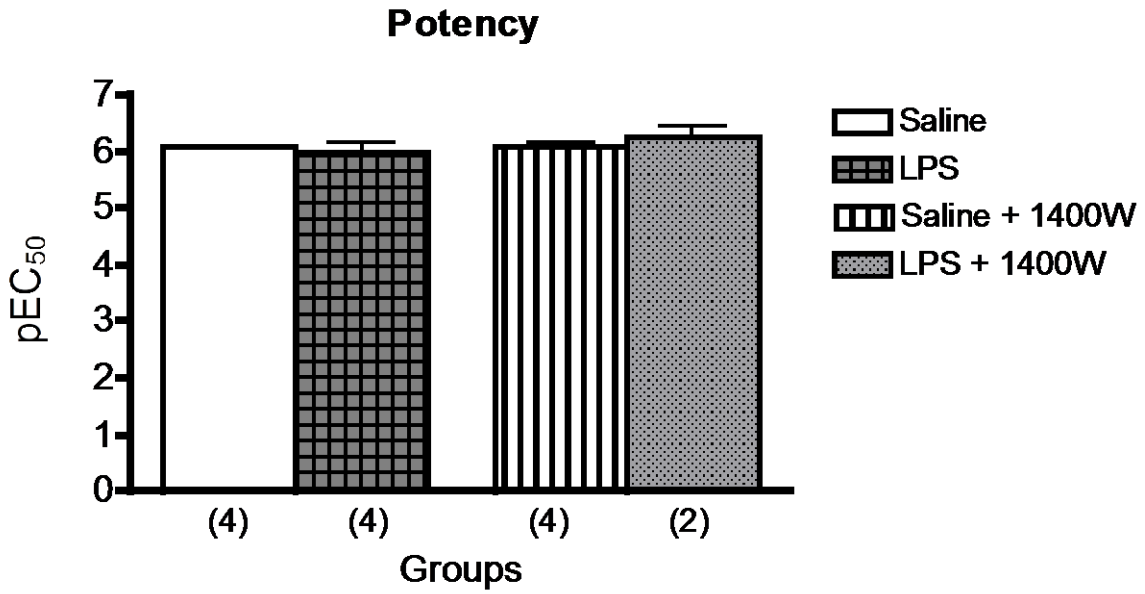


Figure 4.21. The contractile response of Saline and LPS uterine arteries to increasing doses of phenylephrine. Phenylephrine cumulative concentration response curve (A). The pEC_{50} (B). The E_{max} (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

A)


Contractile Response of Uterine Arteries to Phenylephrine
Saline
LPS
Saline + 1400W
LPS + 1400W
22.5
17.5
12.5
7.5
2.5
-2.5
Tension (mN)
-8
-7
-6
-5
log[Phenylephrine]

B)



C)

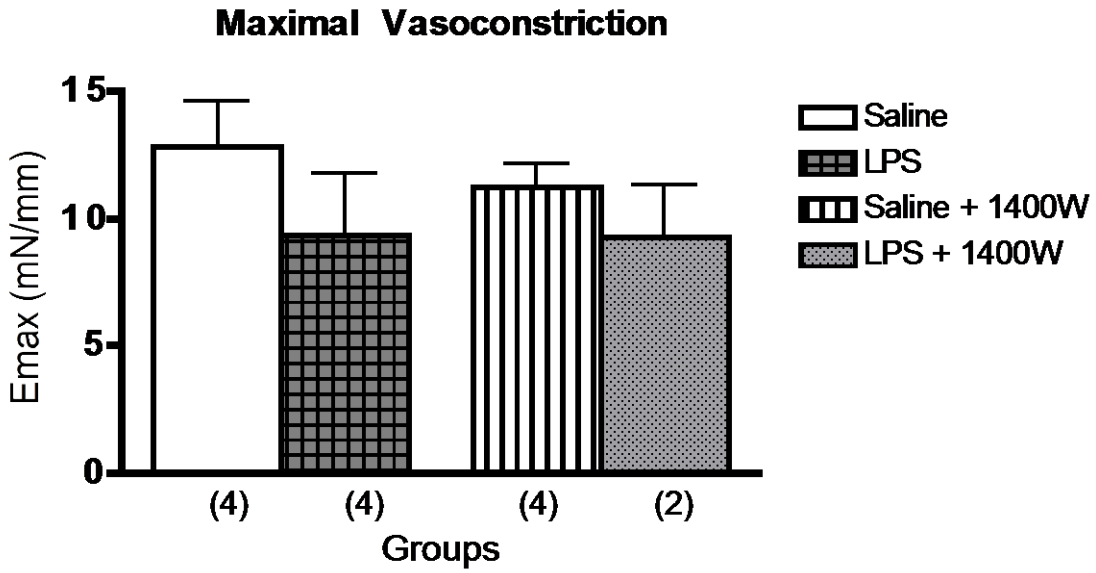
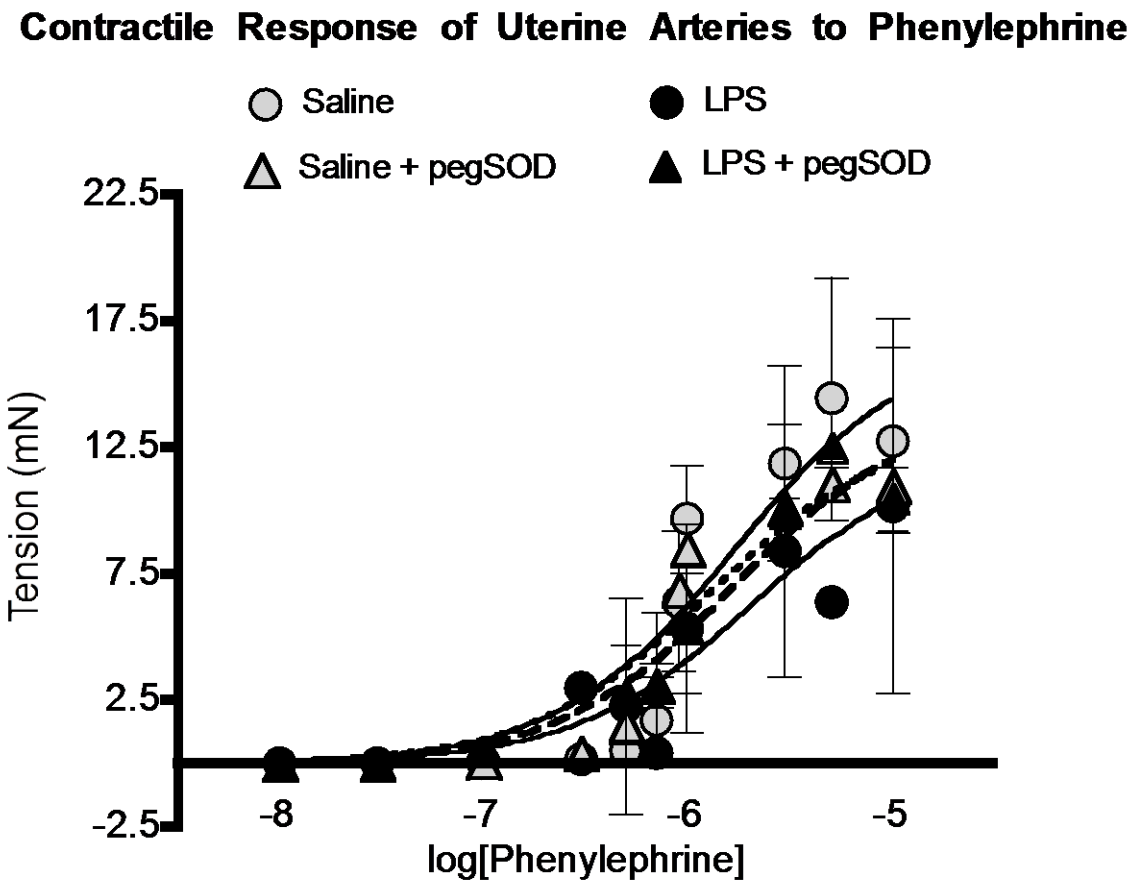
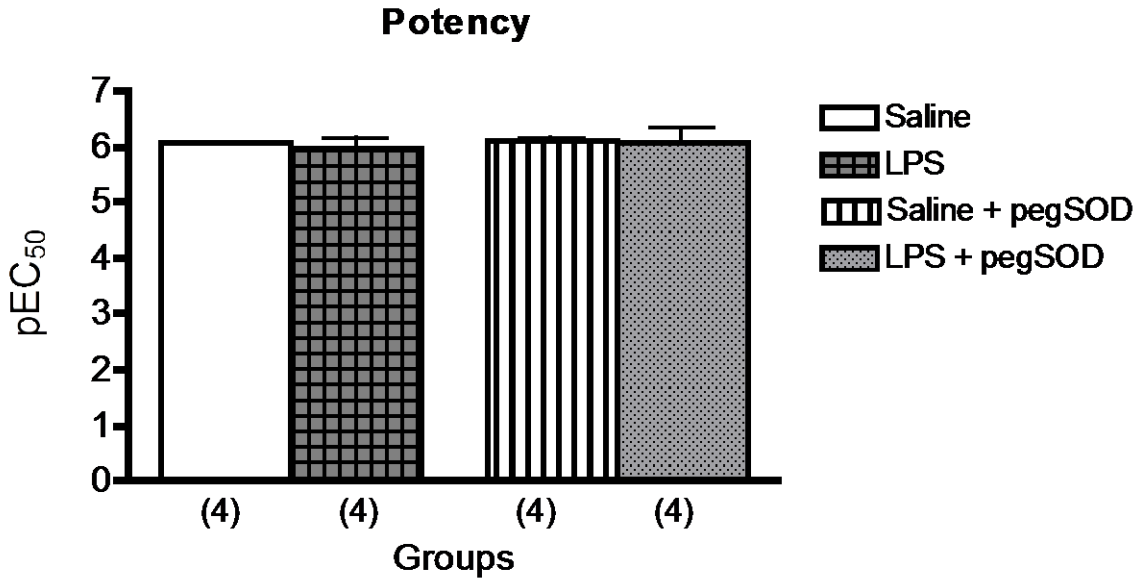


Figure 4.22. The contractile response of Saline and LPS uterine arteries, incubated with the inhibitor 1400W ($1x10^{-6}$ M), to increasing doses of phenylephrine. Phenylephrine cumulative concentration response curve (A). Following incubation with 1400W, no differences were observed in the pEC_{50} (B) or E_{max} (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

A)


Contractile Response of Uterine Arteries to Phenylephrine
Saline
LPS
Saline + pegSOD
LPS + pegSOD
22.5
17.5
12.5
7.5
2.5
-2.5
Tension (mN)
-8
-7
-6
-5
log[Phenylephrine]

B)



C)

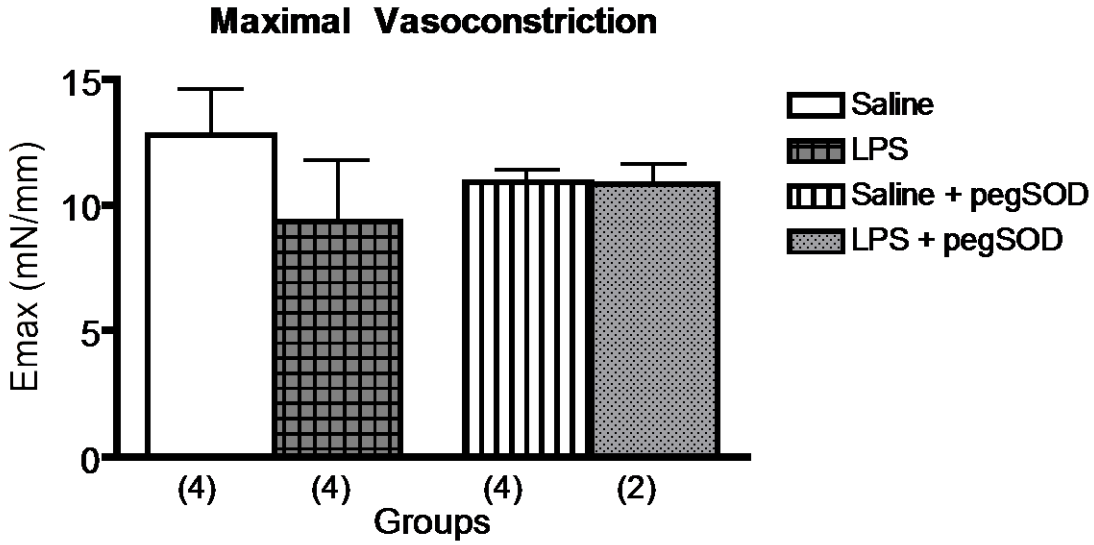
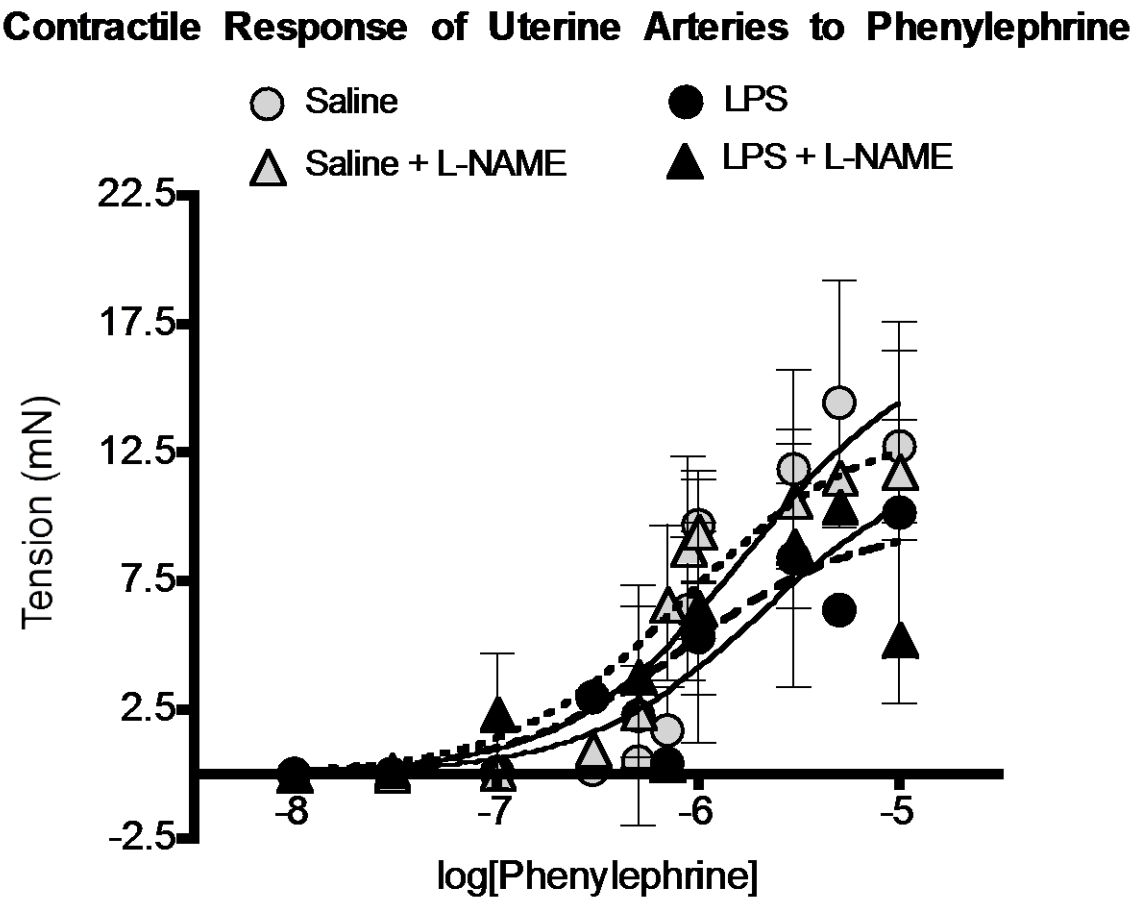
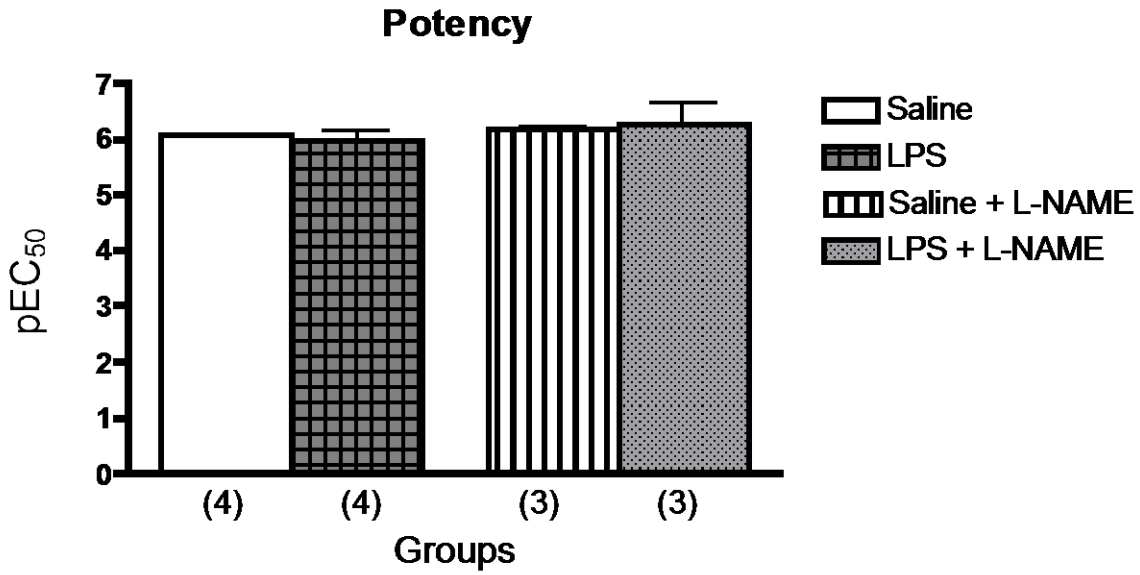


Figure 4.23. Contractile response of Saline and LPS uterine arteries, incubated with pegSOD (50 units/ml), to increasing doses of phenylephrine. Phenylephrine cumulative concentration response curve (A). Following incubation with pegSOD, no differences were observed in the pEC_{50} (B) or E_{max} (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group

A)


Contractile Response of Uterine Arteries to Phenylephrine
Saline
LPS
Saline + L-NAME
LPS + L-NAME
22.5
17.5
12.5
7.5
2.5
-2.5
Tension (mN)
-8
-7
-6
-5
log[Phenylephrine]

B)



C)

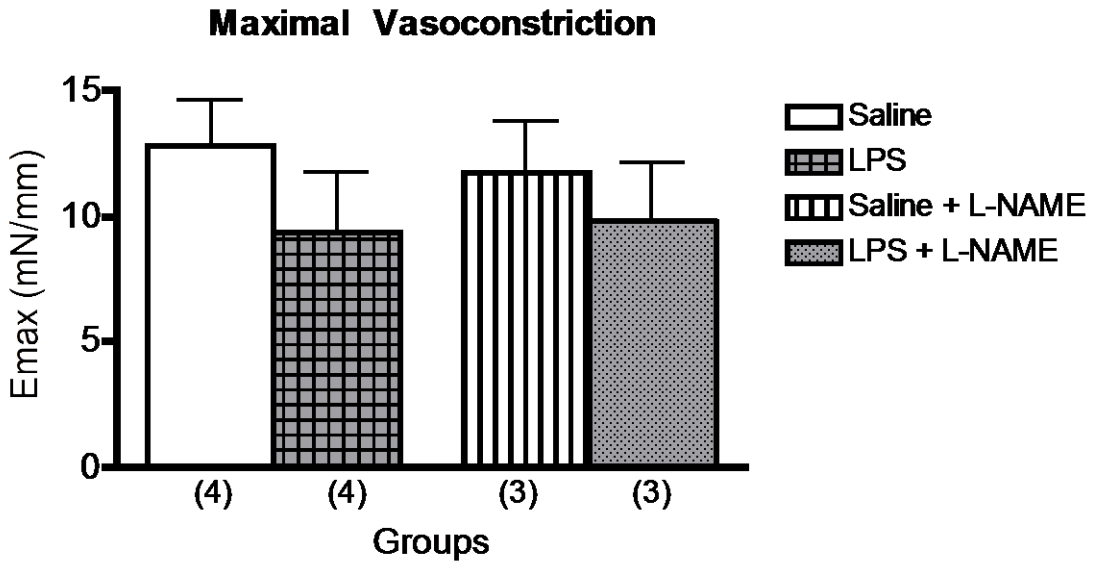
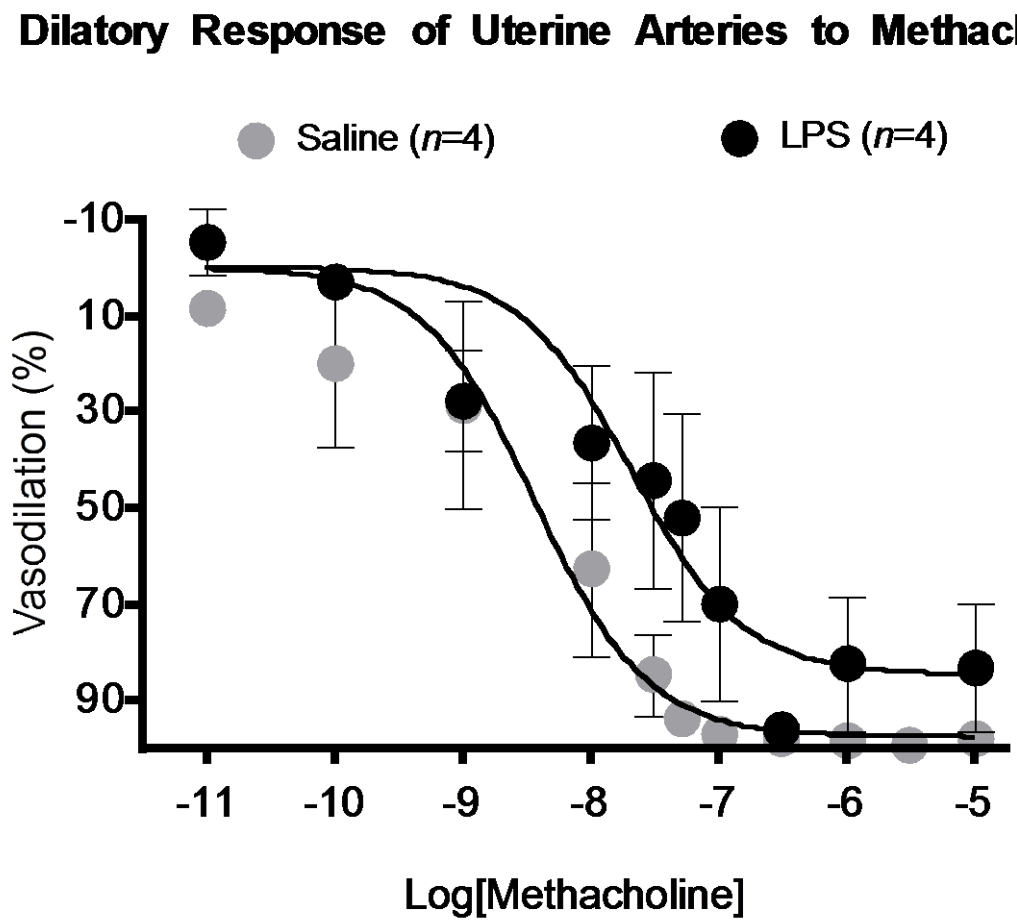
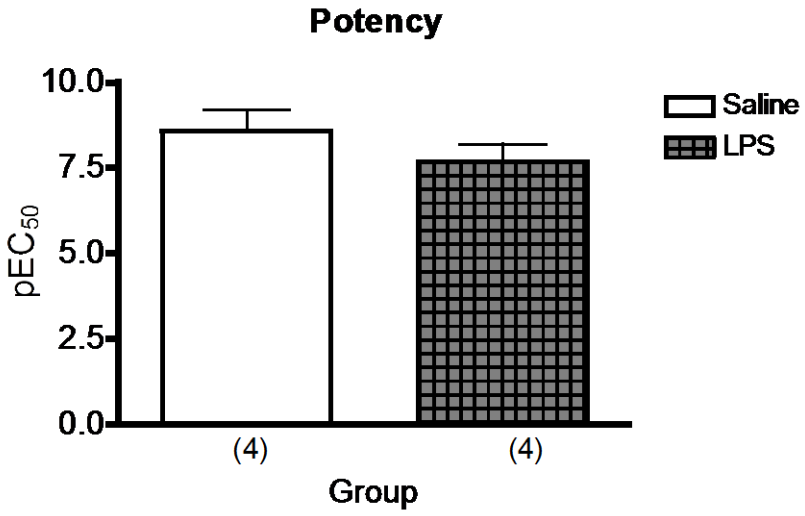


Figure 4.24. The contractile response of Saline and LPS uterine arteries, incubated with the inhibitor L-NAME ($1x10^{-4}$ M), to increasing doses of phenylephrine. Phenylephrine cumulative concentration response curve (A). No differences were observed for pEC_{50} (B) or E_{max} depicted as maximal vasoconstriction (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

A)


Dilatory Response of Uterine Arteries to Methacholine
Saline (n=4)
LPS (n=4)
Vasodilation (%)
-10
10
30
50
70
90
-11
-10
-9
-8
-7
-6
-5
Log[Methacholine]

B)



C)

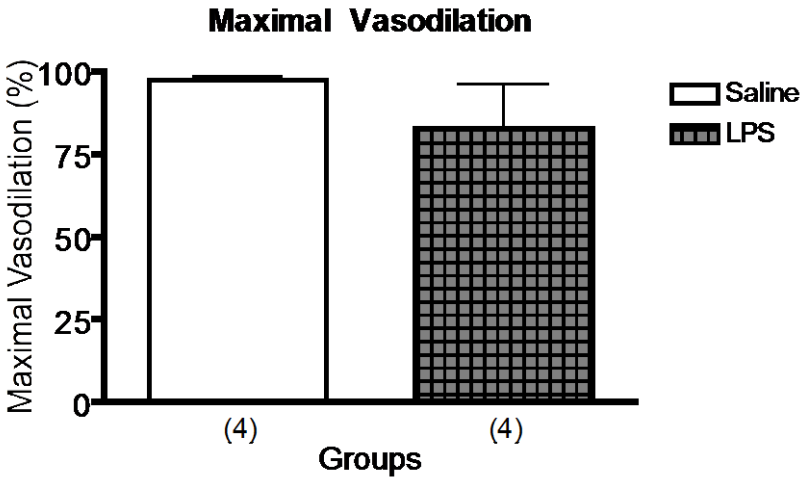
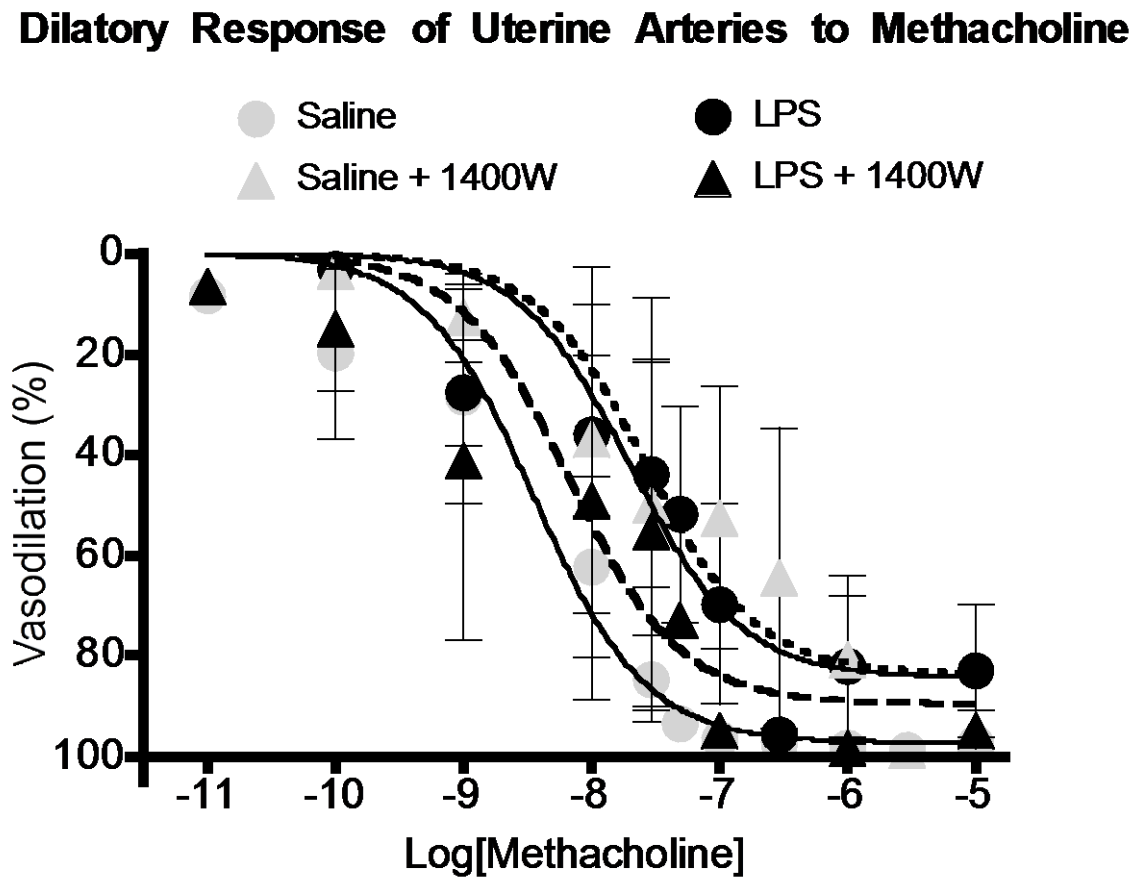
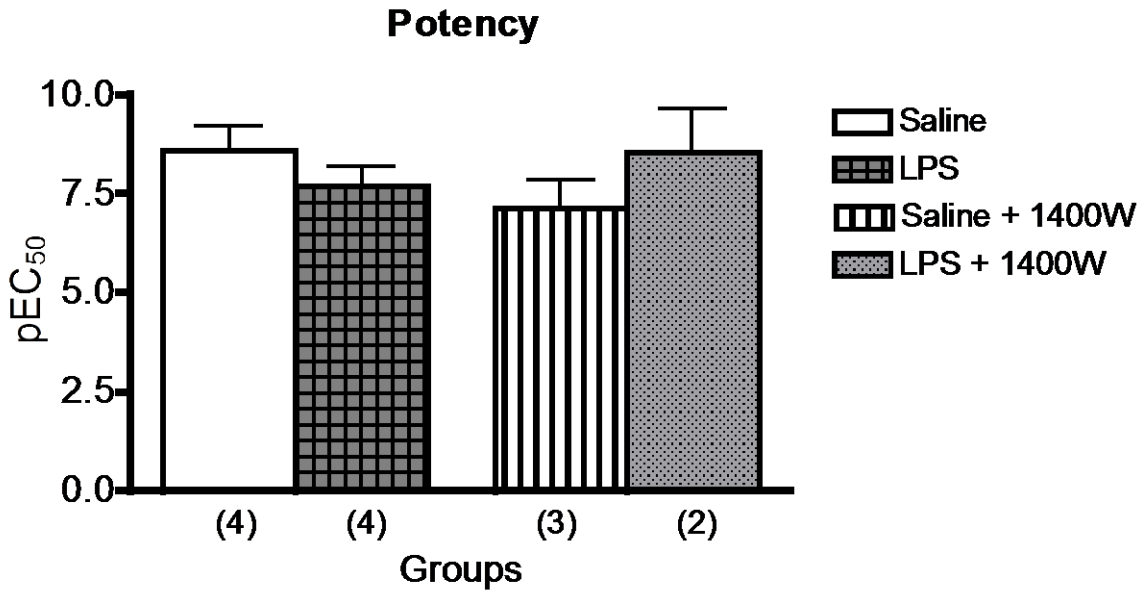


Figure 4.25. The relaxation response of Saline and LPS uterine arteries to increasing doses of methacholine. Methcholine cumuative dose response curve (A). No differences were observed for pEC_{50} (B) or E_{max} (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

A)


Dilatory Response of Uterine Arteries to Methacholine
Saline
LPS
Saline + 1400W
LPS + 1400W
Vasodilation (%)
0
20
40
60
80
100
-11
-10
-9
-8
-7
-6
-5
Log[Methacholine]

B)



C)

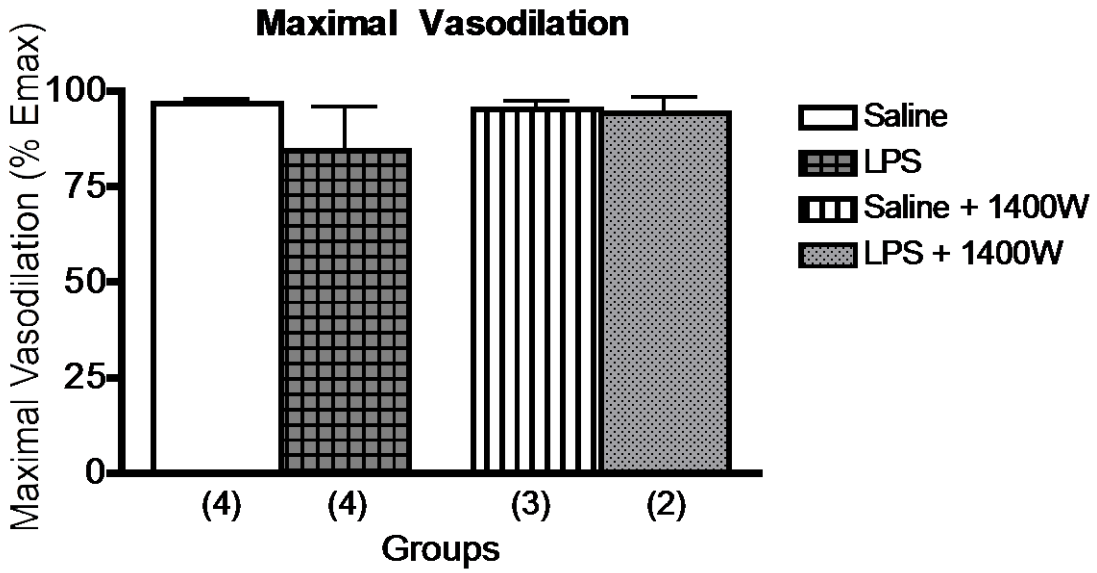
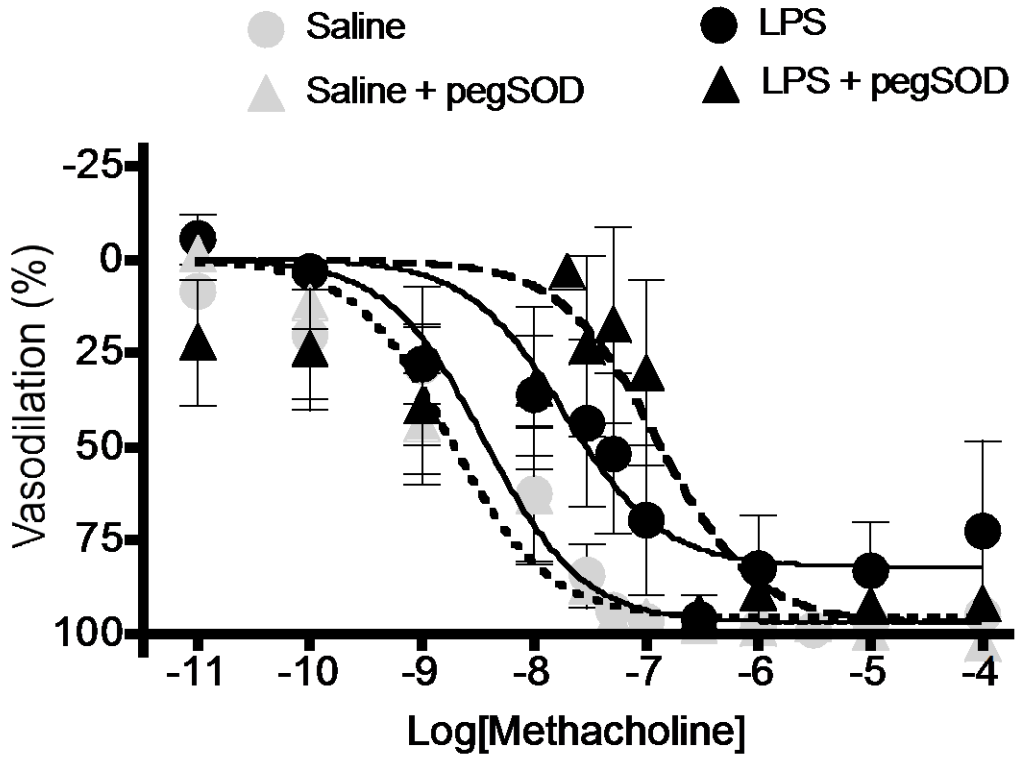


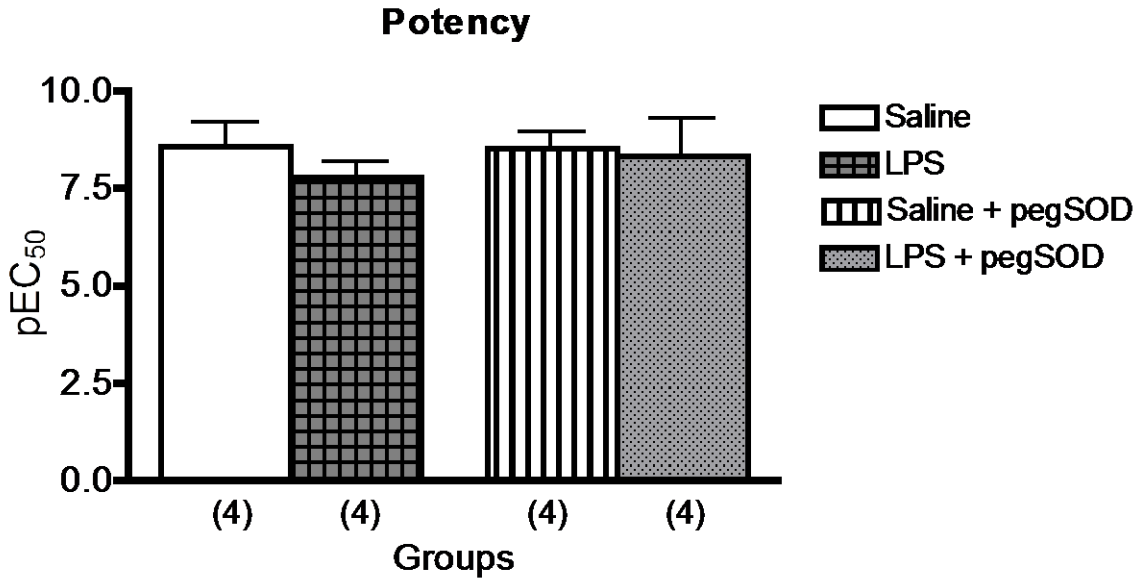
Figure 4.26. The relaxation response of Saline and LPS uterine arteries, incubated with the inhibitor 1400W (1x10-^{6} M), to increasing doses of methacholine. Methacholine cumulative dose response curve (A). No differences in relaxation were observed following incubation with

1400W in pEC_{50} (B), or E_{max} (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

A)


Dilatory Response of Uterine Arteries to Methacholine
Saline
Saline + pegSOD
LPS
LPS + pegSOD
Vasodilation (%)
-25
0
25
50
75
100
-11
-10
-9
-8
-7
-6
-5
-4
Log[Methacholine]

B)



C)

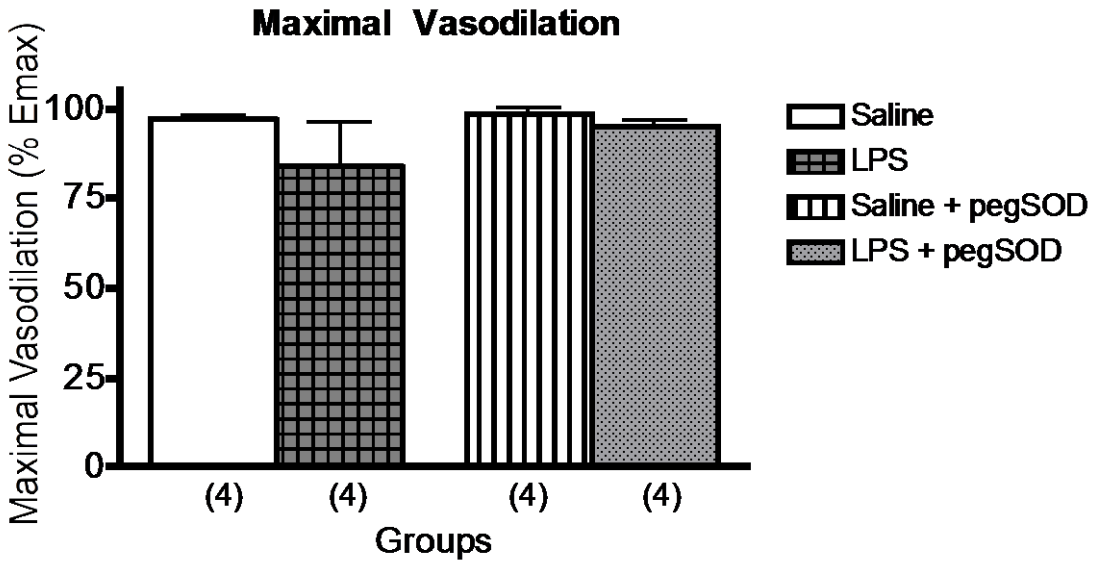
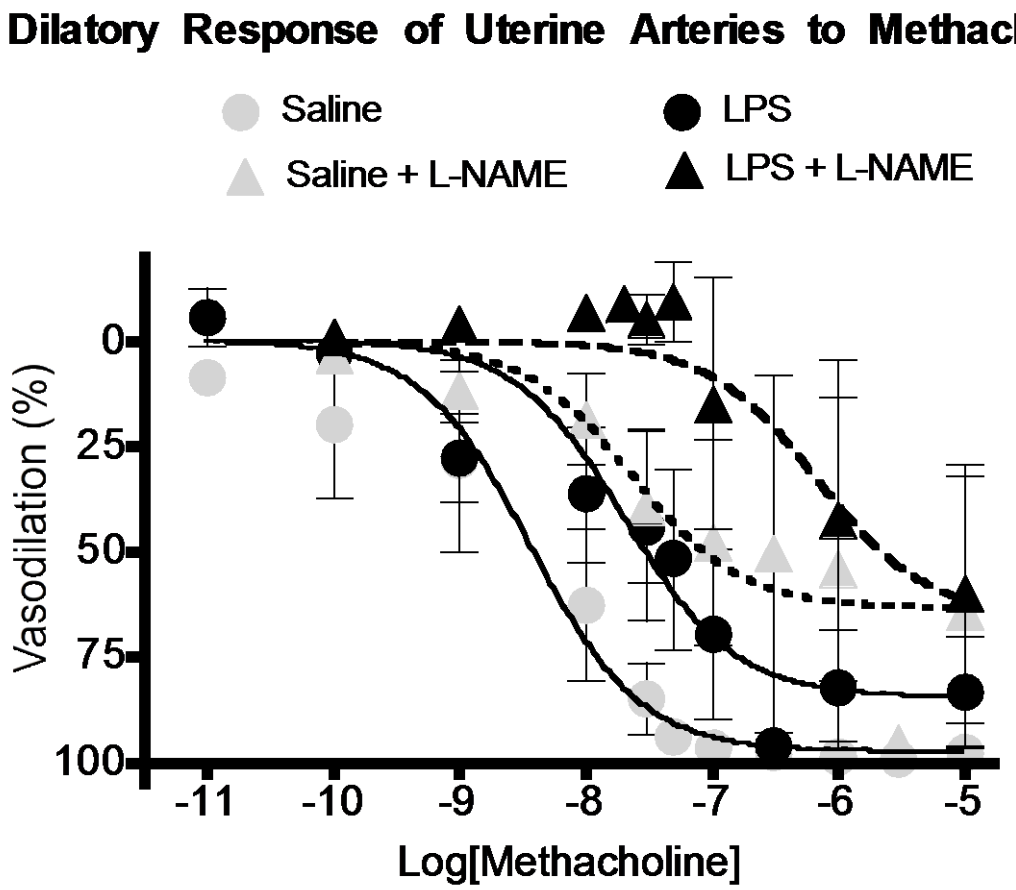
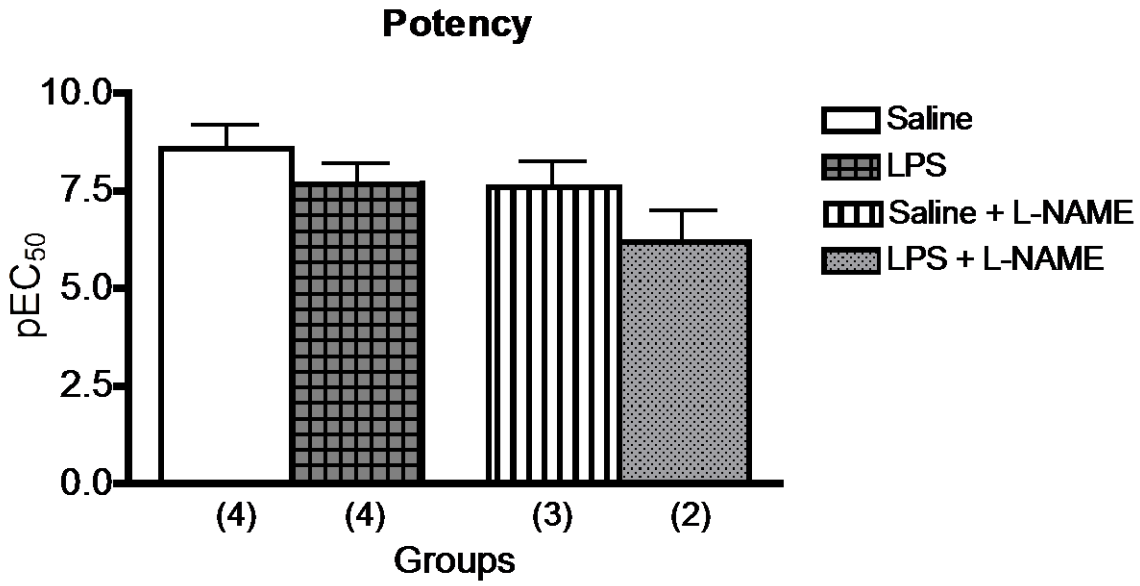


Figure 4.27. The relaxation response of Saline and LPS uterine arteries, incubated with pegSOD (50 units/ml), to increasing doses of methacholine. Methacholine cumulative concentration response curve (A). Following incubation with pegSOD, no differences were observed in pEC_{50} (B) or E_{max} (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

A)


Dilatory Response of Uterine Arteries to Methacholine
Saline
LPS
Saline + L-NAME
LPS + L-NAME
Vasodilation (%)
0
25
50
75
100
-11
-10
-9
-8
-7
-6
-5
Log[Methacholine]

B)



C)

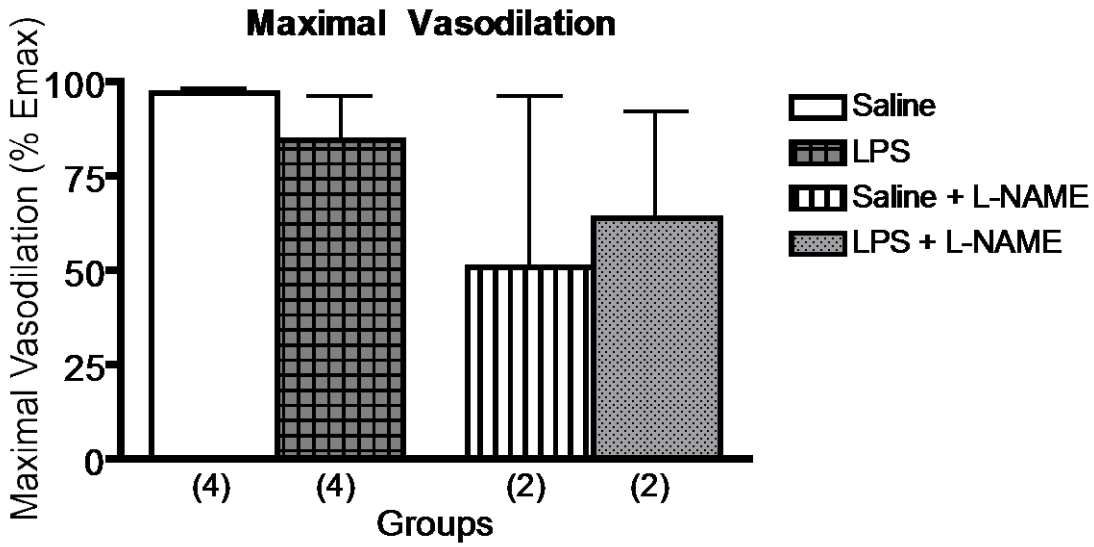


Figure 4.28. The relaxation response of Saline and LPS uterine arteries, incubated with the inhibitor L-NAME ($1x10^{-4}$ M), in response to increasing doses of methacholine. Methacholine cumulative concentration response curve (A). Following incubation with L-NAME, no differences were observed in pEC_{50} (B) or E_{max} (C). Data are presented as mean ± SEM. The numbers in brackets on the x-axis represent the *n* numbers for each group.

5. DISCUSSION

The incidence of CP has not declined over the past decades, despite improvements in maternal and fetal medicine. [1] CP arises from antepartum insults such as chorioamnionitis, which leads to an unfavourable *in utero* environment resulting in a FIR. [81,281] Chorioamnionitis can result in prematurity and intrauterine growth restriction, which are also risk factors for CP. [22,23,27,39,60,282,283] Unfortunately, no preventive therapies exist in part due to the complexity of the insult and also because current pharmaceutical interventions used for adult ischemic brain injury can have harmful effects on the developing brain. [206,284] This has led to the investigation of the possible neuroprotective properties of BrSp.

Previous studies have found beneficial effects of BrSp in rats. In a study by Noyan-Ashraf et al., spontaneously hypertensive female rats (n=6/group) given BrSp (200 mg/day) exhibited increased levels of reduced glutathione and phase II enzymes, and decreased inducible nitric oxide synthase, nitrosylated proteins, and NF-κB p65/IκBα ratio, indicating decreased oxidative stress and inflammation. [231] In addition to the positive effects of BrSp in pregnant females, the offspring also benefited (n=3-6/group). [231] Offspring from dams fed the BrSp diet showed the same beneficial effects, and these outcomes were augmented when the offspring also consumed BrSp. [231] The results suggested a protective property of BrSp during placental insufficiency. [243] Our laboratory has also shown the protective effects of BrSp against reflex delays and white matter injury in a rat model of placental insufficiency. [243] Given these results, we aimed to investigate the effects of BrSp in a model of fetal inflammation, a principle risk factor for the development of CP.

5.1. OBJECTIVE 1

We first evaluated an *in utero* inflammatory model. Several methods are used in the literature and vary with the type of endotoxin used and mode of delivery. [49,285-287] These include injections with live *E. coli*, polyinosinic:polycytidylic acid, and LPS, via intracervical, intraperitoneal, intracerebral, and intravenous routes. Animal models of *in utero* inflammation also vary in species, strain, doses, and timing of gestation for administration. Different strains and species elicit different responses and may be physiologically disparate during various stages of gestation and postnatal development. This can lead to results that vary across laboratories and interventions that may not be translatable. Migale et al. found that certain LPS serotypes (0127:B8, 055:B5, 0128:B12, and 0111:B4) were more potent than others in eliciting preterm births. [244] Pregnant mice (*n*=5/group) were given intrauterine injections of phenol extracted LPS (20 μg) and the time to delivery, pup viability, and inflammatory markers were measured. [244] The LPS serotype 0111:B4 induced preterm delivery the earliest and yielded the fewest viable pups, followed by serotypes 055:B5, 0127:B8, and 0128:B12. [244] The 0111:B4 serotype also had the greatest protein expression of phosphorylated c-Jun, a subunit of the inflammatory transcription factor activator protein-1; however, no differences were detected between the groups for the expression of phosphorylated p65, a subunit of NF-κB. [244] Moreover, the precursor-IL-1β protein was highly expressed in mice that received the 0111:B4 serotype compared to all groups; however, only 0127:B8 and 0128:B12 elicited significantly increased levels of IL-1β. Dean et al. found differences on the number of pre-oligodendroglial and immature oligodendrocytes in different strains of rat. [203] The authors assessed oligodendroglial developmental progression in Sprague Dawley, Long-Evans, and Wistar rat pups (*n*=20 pups from 2 litters/group) on PD2

(*n*=5/group), PD3 (*n*=3/group), PD5 (*n*=5/group), PD7 (*n*=5/group), and PD14 (*n*=2/group). [203] Pre-oligodendrocytes were identified with the marker O4+, immature oligodendrocytes with O1+, and mature oligodendrocytes with myelin basic protein for subsequent analyses. [203] The number of preoligodendrocytes per mm^2 area was greatest on PD2 compared to PD5 and 7, and was more abundant in Wistar rats compared to Sprague Dawley and Long-Evans rats. [203] Immature oligodendrocytes showed the reverse, where minimal numbers were present at PD2, and these numbers increased on PD5 and 7. [203] The number of immature oligodendrocytes on PD2 was greatest in Sprague Dawley compared to Long-Evans and Wistar rats. [203] These studies identified that different serotypes of LPS used and the strains selected within an animal model can result in different outcomes, therefore, the animal model must carefully be considered. We chose Long-Evans rats as our animal model with inflammation induced by intraperitoneal injections of LPS (*E. coli* 0127:B8, 200 μg/kg) on E19 and 20 of a 23-day gestation period, as this model has been shown to cause white matter injury. [256-259]

Long-Evans rats were selected due to lower cost, shorter gestational periods, larger litter sizes, ease of handling, and increased ability to perform motor tasks, compared to other strains. [254,288] Whishaw et al. analyzed forepaw movements of Long-Evans (n=6) and Sprague-Dawley (*n*=8) male rats in a behavioural paradigm referred to as single pellet reaching. [254] The reaching movements of the rats were recorded to allow frame by frame analyses. [254] The authors found that both rats performed equally in regards to reaching for and successfully obtaining a food pellet; however, differences arose when the details of the reaching motion were analyzed. [254] Long-Evans rats' movements were quicker whereas Sprague Dawley's were slower and rigid. [254] Furthermore, their pigmented eyes result in better visual acuity compared to albino strains. [255]

Rodents also give birth to offspring that are altricial, reflecting a neurodevelopmental stage that is equivalent to premature infants at 24 – 28 weeks gestation; the age at which premature human infants have the highest risk of being exposed to *in utero* inflammation and developing CP. As CP is predominantly a motor disorder, Long-Evans rats were determined to be the most appropriate.

LPS from *E.coli* was chosen as the immuno-stimulant. LPS is the endotoxin found in Gram-negative bacteria. Ascending infection leading to chorioamnionitis is often caused by Gram-negative bacteria such as *Mycoplasma* and *Ureaplasma*. [60,289,290] The dosage used was based on previous studies that demonstrated white matter injury following LPS administration. [258,259] This dosage of LPS was chosen to be given near the end of gestation on E19 and E20, similar to the late-stage of vulnerability in human gestation. [107] Endotoxin was chosen over the use of live microbes because studies have shown that the bacteria were not detected, but rather the inflammatory molecules, following post-mortem brain analyses. [56,291] Moreover, LPS is unable to cross the placenta therefore, injuries to the fetal brain resulting from systemic LPS injections are mediated by inflammatory molecules, which supports the 'cytokine hypothesis'. [292,293] Overall, the model reflects a subtle inflammatory model that occurs near the end of gestation in offspring that are developmentally equivalent to premature infants.

Following LPS injections, an initial hypothermic response was observed in the dams, as opposed to the fever response that occurs following infection. [294] Fever is one of the signs of a pro-inflammatory response used to eradicate an infection, and is blunted by IL-10. [295,296] The resultant hypothermic response may be an adaptive mechanism used to protect the fetus;

hypothermia is clinically used to rescue brain injury in newborns with asphyxia. [297,298] Interestingly, Osredkar et al. found that hypothermia was ineffective in rescuing brain damage induced by inflammation-sensitized hypoxic ischemia. [299] This suggests that the use of hypothermia in conditions where the inflammatory response is evoked may not be effective in protecting the brain. Thus, the hypothermic response may be a failed attempt by the dam to rescue the fetuses. Several investigators have identified that the hypothermic response varies with LPS serotype and stage of pregnancy, which suggests a complex interaction between cytokines, cyclo-oxygenase, and NO. [300-304] In this study, LPS + BrSp dams recovered from the hypothermia sooner than the LPS dams, which can be attributed to the anti-inflammatory effects of BrSp. [305] However, the precise role of hypothermia following LPS injections remains to be elucidated.

Not surprisingly, LPS resulted in weigh loss in the dams. [306] A lack of weight gain may result in a disruption of fetal growth, as indicated by smaller litters and reduced birth weights of newborn pups exposed to LPS, due to the limited availability of nutrients from the dam. [151] Interestingly, LPS+ BrSp dams neither lost nor gained weight. This suggests that consumption of BrSp may maintain the weight of the dam by providing some nutrients to promote growth. However, it was not be sufficient to prevent the resorption that caused the smaller litter sizes in both LPS and LPS + BrSp groups. Changes in dietary intake during late pregnancy can disrupt maternal tissue deposition (i.e., protein, minerals, and fat stores), cause dehydration, and alter milk composition, which can result in growth restriction. [307,308,336,337] In addition, brain development can be negatively affected by poor nutrient availability, including changes to cortical thickness, synaptogenesis, and dopamine expression. [309,310] Salas et al. restricted water

availability to pregnant Sprague Dawley rats on E19 for 48 hours and found reduced fetal weights. [311] Naik et al. induced maternal malnutrition using a low protein diet (8%) 45 days prior to impregnation and throughout the lactation period, and maintaining offspring on the same diet as their respective dam, following weaning. [338] Pups in the low protein diet group were smaller compared to controls up until 6 months of age, delayed in the developmental reflex inclined plane test and cliff avoidance, were hyperactive, and had impaired spatial memory. [338] These studies suggests that malnutrition can have a profound effect on the offspring.

Limitations of the maternal analyses were the absence of maternal food and water consumption and milk composition investigations, which may have been altered by LPS and BrSp. Analyses of these parameters, would have provided insight into the nutritional status of the dams. The effect of cross-fostering was also not assessed. Infection during pregnancy can influence maternal behaviour, which can alter brain development in the offspring; this was also a potential confounding factor. [312,313] Moreover, the body mass index of the dams, which is an indicator of health by assessing the level of body fat, was not analyzed because body length was not measured. Pre-pregnant and pregnant rats with high body mass index can be at risk for adverse pregnancies, a confounding factor that was not controlled for. [339] Overall; the possible effects of maternal malnutrition on the outcomes observed in the offspring cannot be discounted.

Pups born from the LPS group were significantly growth restricted, while LPS + BrSp pups were not different from controls. The differences in birth weights may be due to the maternal response to inflammation. For example, maternal weight loss can result in fetal growth restriction. An alternative explanation is that maternal inflammation may have altered uterine

and/or umbilical artery blood flow or vascular dilation, thereby causing a decrease delivery of oxygen and nutrients to the developing fetus. [151,314,315] Furthermore, growth restriction can be caused by impaired placental function, such as an altered expression of glucose transporters that results from inflammation and oxidative stress. [143,148,316] However, growth restriction was no longer observed when LPS dams were supplemented with BrSp. This could be due to both anti-oxidative and anti-inflammatory properties of BrSp, which may have prevented weight loss in the dam and amended artery function and/or placental outcomes. Alternatively, the natural health products found in BrSp may have been sufficient to maintain weight. As mentioned, we were unable to monitor food intake. LPS dams were observed to be lethargic, thus reduced food intake and resultant decreases in micro- and macronutrient uptake may have limited fetal growth.

LPS offspring had delayed developmental reflex acquisition and BrSp supplementation prevented some of these delays, including hind-limb placing and gait. The exact patho-mechanism of inflammation during gestation is unknown, but the immune system does play a role in physiological neurodevelopment. Developmental reflexes reflect postnatal maturation, specifically neural circuitry formation and myelination. Alterations in immune regulation can disrupt normal progression of brain development, resulting in abnormal cortical wiring, functioning, and a lack of myelination, all of which could cause neurodevelopmental reflex delays. The battery of reflexes tested was adapted from Fox and Lubics. [261,262] Both forelimb and hindlimb grasps (palmar and plantar grasps, respectively) are facilitated by spinal reflexes. [317] Hindlimb placing (plantar reflex) reflects maturation of the corticospinal tract. [318] Cliff avoidance, righting, and accelerated righting involve integration and communication between sensory input (vibrissae and vestibular, respectively) and motor output. [319,320] Gait is associated

with corticospinal tract maturation and myelination. [321] Auditory startle assesses the assimilation of sensory (acoustic) stimulation and synaptic connections to produce the appropriate startle reflexes. [322] Posture involves appropriate cortical-spinal/spinal-cortical projections and muscle innervations by neurons. [323,324] *gamma*-Aminobutyric acid A receptor maturation, is suggested to be involved in eye opening. [325] LPS pups were delayed in acquiring hindlimb placing, cliff avoidance, gait, and accelerated righting. These delays suggest that *in utero* inflammation disrupted normal cortical wiring and myelination. BrSp supplementation was able to prevent some of these deficits. LPS + BrSp female pups exhibited the righting reflex earlier than LPS females. In addition, BrSp resulted in earlier eye opening, suggesting a possible influence of BrSp on the maturation of the *gamma*-aminobutyric acid inhibitory system. The effects of BrSp in preventing developmental delay may be due to anti-inflammatory effects that maintain the appropriate balance of cytokines necessary for brain development. A confounding variable in these analyses is weight. LPS pups did not display catch up growth at PD7, however, by PD21, the somatic growth of the pups was no longer different. Thus, it is possible that some of the reflexes may be confounded by weight itself. As such, LPS pups may not have sufficient muscle strength to perform certain reflex tasks such as righting.

Maternal inflammation has been associated with anxious behaviour in offspring, most likely due to elevation of pro-inflammatory mediators during gestation. [326,327] Anxiety and exploratory behaviour in the offspring on PD21 were analyzed with the open field test. Anxiety and exploratory behaviour was measured as the number of squares crossed, rearing, head-lifts, number of times urinated, defecated, grooming, and time spent in the center of the open field box. We did not detect differences in rearing and head-lifts, however LPS pups were observed to

ambulate less and groom more compared to other groups. Grooming releases endorphins, a possible coping mechanism used in stressful situations. [328,329] LPS + BrSp pups groomed less and spent more time in the center of the open field box. This suggests that BrSp may have an effect on anxiety behaviour by dampening the inflammatory response during gestation. A possible mechanism that would prevent anxiety like behaviour is the maturation of *gamma*-aminobutyric acid system. An immature or non-functioning *gamma*-aminobutyric acid has been associated with anxiety; if BrSp is able to accelerate this neurotransmitter maturation, it may attenuate the anxiety-like behaviour. [325 330] Further support of this hypothesis comes from seizure studies, where cytokines have been implicated in the alteration of *gamma*-aminobutyric acid activity and expression, and Nrf2 influences downstream *gamma*-aminobutyric acid signaling. [331,332]

We did not detect changes in brain volume, ventricular volume, and in the number of fractin positive cells suggesting that cell death is not involved in this model at the time points selected for analyses. This suggests that the inflammatory model may reflect dysmaturation rather than cell death. [49,333] Our results revealed no changes in oligodendrocyte numbers, though a significant loss of myelination was observed in the corpus callosum of both LPS and LPS + BrSp offspring. Back et al. reported that pre-oligodendrocytes are the predominant form of cells vulnerable to antepartum insults between 24-32 weeks of gestation. Injury to these cells causes cell death, which are later replenished by progenitors; however, these newly synthesized pre-oligodendrocytes are unable to mature into myelin-producing oligodendrocytes. [36,37,40-43] Our results suggest that this model of FIR resulted in a maturation blockade, leading to the white matter injury observed. Furthermore, a lack of myelination may partially explain a delay in developmental reflexes, as brain tracts for appropriate reflex acquisition require proper

myelination. BrSp did not rescue the decrease in myelin basic protein immunodensity. It did, however, increase 2',3'-cyclic-nucleotide 3'-phosphodiesterase levels such that they were not different from controls (discussed in objective 2). This suggests that the protective properties of BrSp may in part, be mediated through rescue of the myelin-associated enzyme. Overall, the protective properties of BrSp against white matter injury require further investigations.

Our behaviour and pathological analyses were limited to PD21, thus any changes in behaviour and myelination into adulthood cannot be concluded. Another limitation is that other parameters of white matter integrity such as diameter of myelinated axons, width and compaction of white matter tracts, and functional efficacy were not assessed. Furthermore, the potential risks of BrSp were not investigated. Health Canada does not recommend consumption of raw sprouts during pregnancy because it may be contaminated with bacteria. [334] These bacterial pathogens, such as *E.* coli or salmonella, causes food poisoning, dehydration, preterm births, miscarriages, and infections. Moreover, it is also possible for dietary supplementation of BrSp in the diet to produce unwanted side effects. For example, consumption of fava beans in individuals with glucose-6-phosphate dehydrogenase deficiency results in hemolysis. [335] In addition, the components of BrSp may be variable due to different growth conditions such as temperature, lighting, and handling. This poses a complication for translating BrSp dietary supplementation in humans. However, regulations, guidelines, and protocols have been implemented to prevent the contamination of seeds and regulate the processing of BrSp. This approach offers an advantage for translating BrSp to the human population.

5.2. OBJECTIVE 2

The next objective was to identify the underlying pathophysiologic mechanisms associated with *in utero* inflammation and determine whether BrSp can prevent these alterations, thereby protecting the fetus against developmental delay, behavioural deficits, and white matter injury. We hypothesized that both the placenta and fetus would mount an inflammatory response in response to LPS, which would alter neurotrophic production, and disrupt brain development (i.e., neuronal growth and synaptogenesis, glial maturation). These changes were hypothesized to lead to reflex and behaviour anomalies in the offspring. It was hypothesized that BrSp would prevent upregulation of pro-inflammatory cytokines, which would impede downstream changes in neurotrophic factors and brain development. In earlier experiments, BrSp consumption in the LPS group did not restore myelin basic protein expression, thus, BrSp may influence cytokine and neurotrophic factor production, as an alternative mechanism to restore normal functioning.

Placental and fetal weights were recorded following tissue extraction. No differences in groups in placental weights were detected on E19 or E22. Interestingly, on E19, fetuses exposed to the BrSp diet had higher weights and by E22, fetuses exposed to LPS weighed less than the controls. It is possible that consumption of BrSp provided additional nutrients to the fetus, thereby facilitating growth. By E22, it was observed that LPS had caused a reduction in weight gain, possibly through decrease nutrient supply to the fetus as a result of impaired utero-placental functioning. Furthermore, changes to the fetal/placental weight ratio suggest placental morphological and/or functional abnormalities, [340,341] however; we did not detect differences.

Contrary to our findings, Cotechini et al. detected changes to placental weights. [316] The protocol used in their study involved a series of LPS injections to pregnant Wistar rats (*n*=13) on gestational day13.5 (10 μg/kg), 14.5 (40 μg/kg), 15.5 (40 μg/kg), and 16.5, and placental morphometrics were compared to those of Saline injected dams (*n*=13) on gestational day 17.5. [316] The authors found that the placental weights, major axis to minor axis length ratio, and fetal viability were significantly less in LPS groups compared to Saline. [316] This dosing protocol may induce a stronger inflammatory response earlier in gestation, when the placenta is more sensitive to inflammatory environments, leading to altered placental morphometrics. [316] A study by Girard et al. using magnetic resonance imaging analysis found functional changes in the placenta following LPS exposure. [148] Pregnant Lewis rats were injected with LPS (200 μg/kg) or saline (*n*=3-6/group) from E18-20, every 12 hours. [148] The authors found decreased contrast agent accumulation, perfusion, and clearance rates, whereas administration of IL-1 receptor antagonist was able to rescue deficits in placental function. [148] The difference in results may be due to the ability to more easily detect functional abnormalities with magnetic resonance imaging analysis, which is more sensitive than measuring weights and ratios. [148] Alternatively, it is possible that this dosing protocol may not induce gross placental damage.

On E19, we did not detect differences in the number of viable pups between the groups. However, by E22 there was a significant reduction in the number of viable pups in the LPS and LPS + BrSp group. Not surprisingly, LPS has been shown to induce fetal death/resorption, however, BrSp was unable to prevent fetal demise at these time-points. [151] Moreover, no differences in crown rump length were detected on E19, however, by E22, there was a reduced crown rump length in both LPS and LPS + BrSp groups. Following birth, the LPS + BrSp pups

were larger than the LPS pups. An explanation for these observations is that inflammation may induce altered uterine artery and placental functioning, leading to a reduced capacity to deliver nutrients to the growing fetus, resulting in growth restriction earlier in gestation since this was not supported by the results obtained from objective 3. This inflammatory response may be dampened by E22, while the ongoing effects of BrSp improve the growth of the fetus in LPS + BrSp pups, possibly by increasing nutrient supply and/or preventing loss of blood flow and uterine artery vasodilation.

Studies on the passage of cytokines from maternal to fetal circulation via the placenta are limited, but have shown that IL-6 is transferable while IL-1α and IL-1β are limited. [79,80] Cytokine mRNA expression was measured in placentas and fetal brains. *In utero* inflammation induced an upregulation of pro- and anti-inflammatory cytokines in the placenta on E19; however, the increased expression was no longer significant by E22. On E22, there was a significant main effect of diet where IL-6 expression was reduced in both Saline + BrSp and LPS + BrSp groups. These results are in agreement with the existing literature. Girard et al., found a significant upregulation of placental IL-1β, IL-1Ra, IL-6, and TNF-α following inflammation compared to control placentas. [148] Upregulation of placental IL-1β, TNF-α, and IL-6 mRNA was also detected after 100 μg/kg of LPS, half the dosage used in the current studies. [342] BrSp have been shown to exert anti-inflammatory effects, which may in part explain the reduction of IL-6 mRNA expression in the BrSp groups. [230,231] This is of particular interest since FIR is defined by an elevation of systemic IL-6. [81,281] Thus, BrSp may be effective in preventing elevation of IL-6 in the placenta and possible passage of the cytokine into the fetus.

In fetal brains, there were no changes in IL-1β, TNF-α, or IL-6 cytokine mRNA levels at E19, however IL-10 was higher in the LPS + BrSp group. On E22, no significant upregulation of IL-1β (spanning exons 5 and 6), TNF-α, IL-6, or IL-10 were observed; however, IL-1β (spanning exons 2 and 3, which codes for pro-IL-1β) was significantly upregulated in the LPS group compared to the other three groups. This suggests that fetal inflammation and brain injury may be mediated predominantly by IL-1β whereas BrSp may 'prime' the fetal brain with an upregulated IL-10 response that deters the increase in IL-1β. In agreement with our findings, in a mouse model of *in utero* inflammation via polyinosinic:polycytidylic acid administration, only IL-1β was increased in the adult offspring. [343,344] Favrais et al. reproduced white matter injury by injecting Swiss male mice with IL-1β twice a day on PD1-4 and once on PD5. A second group was injected twice a day on PD6-9 and once on PD10. [92] On PD35, magnetic resonance and diffusion tensor imaging revealed altered diffusivity and fractional anisotropy, which indicated functional abnormalities in the white matter tracts. [92] Mice injected on PD1-5 performed worse on object recognition and location memory behavioural tests compared to controls and those exposed to LPS on PD6-10. [92] White matter injury was observed, evidenced by increased expression of myelin basic protein, proteolipoid protein, myelin associated protein, and decreased 2',3'-cyclic nucleotide 3'-phosphodiesterase. [92] The increase in fetal brain IL-1β mRNA expression following LPS stimulation suggests that the fetus is capable of mounting its own FIR. Interestingly, we detected an upregulation of IL-1β spanning exons 2,3 but not 5,6, thus developmental splicing may contribute to detection of upregulated mRNA expression and brain injury. [345-348] The increase in the IL-1β mRNA was for pro-IL-1β. This may represent a sensitizing mechanism of inflammation for subsequent sub threshold insults such as hypoxic ischemia. It is hypothesized that BrSp may be preventing an increased expression of pro-IL-1β

mRNA, thereby preventing sensitization. We did not observe changes in fetal brain IL-6 expression, contrary to the hypothesis; however, the definition of a FIR refers only to an increase in plasma IL-6. [281]

It is possible that the anti-inflammatory effects of BrSp were involved in reducing pro-IL-1β mRNA expression by preventing NF-κB activation and translocation, which may in part explain the rescue of developmental delays caused by LPS. [305] BrSp may have an effect on DNA transcription through histone modifications and/or methylation, which may in part explain why the expression of pro-IL-1β was not increased in the LPS + BrSp group. [349] In addition, it would be of interest to determine if the increase in pro-IL-1β is translated into the functional protein counterpart, IL-1β, on E22. This would highlight whether or not IL-1β is expressed in a short timeframe that is sufficient to induce reflex anomalies and white matter damage since the protein was not detected on PD1. This would include analyses of downstream cleavage of pro- IL-1β by the inflammasome and MMPs. The inflammasome activates caspase 1, which then cleaves pro-IL-1β into the active IL-1β. [350] Similarly, MMP-2, -3, and -9 can cleave pro-IL-1β into its active constituent. [351]

No differences in PD1 brain protein expression of IL-1β/IL-10 were detected among the groups. However, LPS and LPS + BrSp female fetuses had a reduced brain TNF-α/IL-10 and IL-6/IL-10 protein expression. Interestingly, Saline + BrSp male fetuses had a significantly reduced brain expression of TNF-α/IL-10 (compared to Saline) and IL-6/IL-10 (compared to LPS + BrSp). It is hypothesized that the changes in the ratio of cytokines may reflect altered protein concentrations that would disrupt brain development resulting in the reflex delays

observed. [98] Results of behavioural testing did not reveal differences in reflex and behaviour performance between Saline + BrSp and Saline groups, thus, these changes in cytokine expression may not be detrimental. As mentioned, these results did not suggest cell death but rather maturational changes. An alternative possibility is that BrSp may induce altered cytokine expression that may promote and enhance neurodevelopmental progression, such as neurogenesis and oligodendrogenesis in the Saline + BrSp group. [98] Interestingly, no differences in IL-1β/IL-10 expression were identified, suggesting that the detrimental effects of IL-1β occurred during a narrow time window *in utero* that was sufficient to induce white matter injury and behavioural alterations in offspring. Overall, it was hypothesized that an increase in pro-inflammatory cytokines would occur however, this was not observed. It is possible that cytokine changes are longer present, or different immune mediators may be involved. The precise functional role and contribution of these cytokines to brain development is complex and not fully understood. Further explorations are required to understand the functional role of each cytokine under normal and pathological conditions.

Oxidative stress occurs with inflammation, thus matrix metalloproteinase-2 activity and reduced/oxidized glutathione ratio were evaluated in newborn rat pups. [352] A sex effect was detected for matrix metalloproteinase activity; however, upon separation of males and females, no significant differences were found. The gelatin zymography analyses did not detect matrix metalloproteinase-9 activity, similar to the study by Ranasinghe et al. where developmental expression of matrix metalloproteinase-2 and -9 was assessed in Wistar rat brains, from E18 to PD120. [353] Gelatin zymography and polymerase chain reaction were used to detect enzyme activity and mRNA expression, respectively. [353] The authors found that the pro and active form

of matrix metalloproteinase-2 expression were highest at E18, and decreased as the rat aged. [353] Matrix metalloproteinase-9 was not detected in the developing brain until exposure to a hypoxic-ischemic injury occurred, suggesting an influence of oxidative stress on matrix metalloproteinase-9 induction. [353] Similar to the PD1 cytokine outcomes, the lack of changes is surprising and the role of MMP following fetal inflammation may not be involved at this stage.

As mentioned, BrSp contains SFN, which enters the cell and releases Nrf2. Nrf2 binds to the antioxidant response element promoter region and promotes production of several antioxidant enzymes including enzymes involved with glutathione production and conjugation to reactive species. [354] This emphasizes the important role of glutathione as a defense mechanism and as a downstream effect of BrSp consumption. Therefore, glutathione was selected as a marker of oxidative stress. Sex effects were also detected for glutathione analyses. A significant main effect of Diet was found following reduced/oxidized glutathione analyses where the reduced form was highly expressed in the LPS + BrSp female group. These results suggest that BrSp enhanced production of the antioxidant glutathione to protect the developing brain from oxidative stress, in females. Furthermore, the results suggest that oxidative stress may not be heavily involved at this time point, as no increase in matrix metalloproteinases or decrease in reduced/oxidized glutathione ratio was observed in the LPS groups. During intrauterine development, the fetal rat (E18-E19) has a partial pressure of oxygen of ~22.5 mmHg whereas the brain has ~4.5 mmHg therefore, pro-oxidant production may be limited and the precise contribution of pro-oxidants to development or brain damage in this model is unknown. [355]

During gestation, placental cells provide the fetal brain with neurotrophic factors for appropriate development. [356] The three groups of neurotrophic factors analyzed were BDNF, NGF, and NT-3. These neurotrophic factors have several important roles in brain development such as proliferation, migration, and differentiation. Cytokines are capable of communicating with and regulating neurotrophic factors. [273] Changes in cytokine levels may alter production and/or function of neurotrophic factors. No differences among the groups in placental neurotrophic factor expression were detected on E19 and E22. On E19 in the fetal brain, no differences in neurotrophic factor expression were observed. On E22, there was a significant reduction in fetal brain NGF expression in both LPS and LPS + BrSp groups. This suggests the increase in pro-inflammatory cytokines in the placenta and/or IL-1β may cause a decrease in fetal brain NGF mRNA. Cytokines have been shown to increase expression of the major histocompatibility complex resulting in reduced synaptogenesis, whereas NGF has been shown to reduce the immune complex expression, promoting synaptogenesis. [273,357,358] It is possible that the changes observed may alter synaptogenesis in postnatal brains. Although BrSp consumption was effective in preventing IL-1β elevations in the fetal brain, it was not effective in preventing the loss of NGF on E22. Sulforaphane has been shown to have epigenetic effects and thus, BrSp may exert protective effects on NGF expression at the protein level via posttranslational modifications. [349,359]

The expression of nuclear Nrf2 and cell death was assessed on PD1. No differences were detected in nuclear Nrf2 between the four groups. Sulforaphane may not mediate its protective effects through activation of endogenous antioxidant systems at this time point, despite the increased reduced/oxidized glutathione ratio in the LPS + BrSp group on PD1. Although SFN

has been shown to influence cell cycle and apoptosis, no differences in cleaved caspase 3 or FluoroJade B were detected between the groups, suggesting a lack of cell death at PD1. A possible explanation is that the methods used were not sensitive enough to detect cell death. On PD21, we assessed the number of NeuN positive cells, which is a marker of mature neurons. There were fewer NeuN positive cells in the hippocampus and posterior parietal cortex of Saline + BrSp and LPS + BrSp groups compared to the other groups. BrSp may accelerate developmental programming, eliminating cells that were destined for removal. Earlier pruning of cells may have occurred, since these two groups did not perform significantly differently from one another in most of the reflexes tested, and Saline and Saline + BrSp pups were not different in any behavioural parameters examined, except for eye opening. Similarly, a significant decrease in NeuN positive cells in the anterior caudate putamen region occurred in the LPS groups compared to the Saline groups. Long-term behavioural analyses were not pursued, so whether increases in NeuN positive cells in these regions can cause alterations in behaviour in adulthood remains unknown. [360,361]

Several markers of postnatal brain maturation were assessed to evaluate the effects of inflammation and BrSp on brain maturation. Neurotrophins such as NGF have been shown to induce growth associated protein 43 activation. [362-364] This suggests that inflammation may have an effect on the ability of neurons to develop properly, specifically respond to migratory cues, establish connections for brain wiring, and form synapses for communication, all of which involve proper growth associated protein 43 expression. However, no differences in gap associated protein 43 expression were detected in this study. Similarly, no differences in matrix metalloproteinase activity were detected. Matrix metalloproteinases are also involved in pruning

for development; interpretation of matrix metalloproteinase production should not focus solely on oxidative stress. Changes to matrix metalloproteinase activity may correlate with decreased expression of growth associated protein 43, and therefore cellular maturation. Ranasinghe et al. found that on PD3, matrix metalloproteinase-2 was localized to the cortical plate and subplate regions in Wistar rat brains, thus matrix metalloproteinase-2 activity may be closely associated with neuronal differentiation, such as neurite outgrowth. [353] To identify whether reduction of growth associated protein 43 and NGF influenced synaptogenesis, synaptophysin and syntaxin were analyzed; no differences were observed. In contrast, Giovanoli et al. injected C57BL6/N mice with polyinosinic:polycytidylic acid (5 mg/kg) on gestational day 9 (*n*=8/group) and found a reduction in the postsynaptic protein PSD95 without alterations to microglial numbers or activation states, astrogliosis, along with increased hippocampal IL-1β at adult ages (5 months). [343,344] In a separate study by Giovanoli et al., mice injected with polyinosinic:polycytidylic acid (5mg/kg) on gestational day 17 (*n*=8-10/group) exhibited reduced synaptophysin expression in older age (22 months), without evidence of microglial activation or alterations to cytokine expression. [343] The data suggest that prenatal immune challenge can induce lasting effects in the brain without signs of gliosis or inflammation. A limitation in the current study is that synaptic markers were assessed only up to 3 weeks of age whereas Giovanoli et al. investigated these markers in beginning at one month of age (pubescent) to 22 months of age (aged). [343,344] Overall, placental and fetal inflammation reduced NGF in the fetal brain; however, no other changes to the neuronal maturation markers were identified at the time points evaluated.

Oligodendroglial maturation was assessed with Olig2, a pan-oligodendroglial marker, and 2',3'-cyclic nucleotide phosphodiesterase, a marker of differentiating oligodendrocytes. A

significant decrease in 2',3'-cyclic nucleotide phosphodiesterase expression in the LPS and LPS + BrSp groups was observed. LPS pups also had a significantly lower white matter 2',3'cyclic nucleotide phosphodiesterase immunodensity compared to Saline pups on PD21. Although LPS + BrSp pups were not different from LPS pups, they were also not different compared to their respective control, suggesting that BrSp may have had an effect on oligodendroglia maturation. 2',3'-cyclic nucleotide phosphodiesterase is an indicator of oligodendroglial differentiation and supplementation with BrSp in the LPS group may have promoted differentiation of the cells to myelin producers. In agreement with these findings, myelin basic protein immunodensity was reduced in white matter in both LPS and LPS + BrSp pups on PD21. Although BrSp did not have an effect on myelin basic protein expression, proteolipid protein, or myelin oligodendrocyte glycoprotein, other common myelin proteins were not investigated. In addition, compaction of white matter layers and axon diameter were not assessed, and are influenced by myelination. Furthermore, no differences were detected for Olig2 on PD1, PD7 and PD21, suggesting that oligodendroglial cell death did not occur. It appears that this model of inflammation did not induce oligodendroglial cell death but may have caused dysmaturation, or that oligodendroglial cell death was induced by inflammation but progenitor cells were able to replenish the loss. Dietary BrSp supplementation was able to prevent the loss of mature oligodendrocytes in the LPS + BrSp group.

The expression of activated microglia was assessed on PD1; as others have observed, no changes in expression were caused by *in utero* inflammation. [160,343,344] Similarly, no changes in activated astrocytes were observed among the groups on PD1, 7, or 21. These results were unexpected since inflammation promotes activation of microglia and the protective effects of sulforaphane have been shown to be facilitated through activation of astrocytes, in an effort to

protect neurons against damage. [239] In this study, it is possible that SFN activated astrocytes, thereby enhancing glial fibrillary acidic protein immunodensity, and causing the secretion of factors such as neurotrophic growth factors, as a mechanism of neuroprotection. This may, in part, explain the observed rescue of developmental delays. However, we did not detect astrogliosis thus, a different neuroprotective mechanism of SFN may be taking place.

A limitation to the analyses of mRNA expression in the placenta and fetal brains are the time points selected. This study only examined two time points, the first six hours after the first injection, and the second 36 hours after the last LPS injection. Cytokines have a short half-life, therefore changes in between may have been missed. [342] In addition, cytokine protein levels on E19 and E22 were not assessed. This poses as a limitation because mRNA levels do not always with protein expression, which can be affected by several factors such as microRNAs, successful translation of the mRNA into a protein, formation of a functional protein, etc. [365] The primers used spanned two exons and a single intron; thus, contamination of the sample with DNA would not be transcribed. [365] Moreover, only a single primer was used for each target, therefore other splicing variants encoding different protein isoforms may not been detected. [365] Not to mention, only the pro-form of IL-1β was assessed thus, the precursor form of other cytokines such as TNF-α will need to be addressed. During tissue preparation, the fetal brain tissue was homogenized altogether, therefore different cytokine mRNA expression within subsets of cells cannot be accounted for and differences may have been diluted. The cytokine analyses were limited to the placenta and fetal brains. The study did not address the presence of immune cells such as macrophages and leukocytes, as well as inflammation of the chorion and amnionitic membranes, umbilical cord, and the amnionic fluid. [60] Evidence for the aforementioned would

have provided additional supporting evidence for *in utero* inflammation following exposure to LPS and possible targets of BrSp.

In regards to oxidative stress, reactive oxidative and nitrosative species were not directly measured. The analyses of glutathione only provided indirect evidence of oxidative stress. Moreover, the production of antioxidant enzymes such as heme oxygenase and glutathione peroxidase by BrSp was not evaluated, thus, the antioxidant properties of BrSp cannot be concluded and requires further investigation. [252] However, the lack of differences in Nrf2 expression suggests the BrSp may be protecting the fetal brain through a different mechanism. [366] Different neurotrophic factors and maturational markers exists, therefore the ones selected for assessment may not have been proteins that are involved following fetal inflammation. Furthermore, the investigations were conducted on pre-weaned animals, such that changes to neuronal maturation and function may not be detected until later ages.

Moreover, small sample sizes (representing litters) were used in the experiments. Some of the experimental tests may not have reached sufficient power to detect a true and significant difference. The analyses of the data were performed using two-way analysis of variance. A limitation using this statistical methodology was the small litter sizes, which may have not presented with a normal distribution (Gaussian distribution). A normal distribution follows a bell shaped curve, where the mean lies in the center. Within the experiment, 68% and 95% of values would be found one and two standard deviations away from the mean, respectively. Following additional experiments to increase litter sizes, if the results do not follow a normal distribution, then a non-parametric test such as the Kruskal-Wallis or Friedman tests should be considered.

Overall, the changes observed in the second objective were much more subtle than expected, due to the absence of significant changes to axonal growth (albeit a trend towards significance), astrogliosis, and synaptogenesis in this model. These results suggest that there may be no alterations to the maturation of neurons, and this model may reflect deficits that are confined to cells associated with myelination. Alternatively, this inflammatory model may be severe enough to result in brain damage. Another possible explanation is that this model may reflect a sensitizing model, where the developing brain is sensitized to subsequent subthreshold injurious events. The mechanism of fetal inflammation still requires further investigation due to the complexity and spectrum of damage seen in the literature.

5.3. OBJECTIVE 3

Vascular dysfunction is implicated in maternal inflammation (i.e., chorioamnionitis) and has been characterized as enhanced vasoconstriction and reduced vasodilation, mediated by proinflammatory cytokines such as IL-6 and TNF-α. [130,367] Increases in blood cytokines can cause endothelial dysfunction, which can result in intrauterine growth restriction. [342] Thus, the next objective was to identify whether this model of inflammation causes maternal uterine vasculature dysfunction, resulting in growth restriction. The aims were to identify whether: 1) uterine and umbilical arterial blood flow was altered following inflammatory challenge, 2) LPS enhanced vasoconstriction and reduced vasorelaxation, and 3) the pathomechanism involved could be inhibited by reducing inflammation and oxidative stress via BrSp consumption. [368] The data obtained permitted assessment of altered vasculature in the model, and were intended to assess the capacity of BrSp dietary supplementation to rescue these deficits.

Prior to analysis of *ex vivo* artery function, blood flow in the uterine and umbilical arteries was investigated. It was speculated that inflammation would increase vasoconstriction, which would subsequently reduce blood flow and lead to the fetal growth restriction that was observed. Interestingly, analysis of blood flow in both the uterine and umbilical arteries revealed no changes in pulsatility or resistance index between Saline and LPS dams. At E21, there was no impedance to blood flow, therefore we did not pursue analysis of the BrSp groups. These results were unexpected and contrary to the hypothesis. In contrast to the results obtained, Renaud et al. reported impaired uteroplacental blood flow in response to inflammation. [151] Pregnant Wistar rats were injected with 100 μg/kg of LPS (serotype 0111:B4) midgestation (E14.5) and a significant increase in uterine resistance index was observed, paralleled by a decrease in blood flow. [151] Girard et al. also found a reduction in perfusion within the placenta, indicative of altered uteroplacental and umbilical placental blood flow. [143] In these studies, changes to blood flow were analyzed immediately after LPS injection. In the current study hemodynamics were investigated on E21, a day after the last injection. The lack of changes identified may be attributed to the time point chosen to evaluate vascular function. In addition, the LPS serotype selected may not have as robust effects as the ones used in the studies, leading to different outcomes.

Proinflammatory cytokines were hypothesized to impair endothelial function by suppressing vasodilator-signaling pathways, while enhancing constrictor pathways. Inflammation involves activation of NF-κB, which leads to increased expression of NO via induction of inducible nitric oxide synthase, as well as the generation of proinflammatory

cytokines and pro-oxidants (i.e., superoxide anion, activation of cyclooxygenase-2 by inducible nitric oxide synthase, and nicotinamide adenine dinucleotide phosphate oxidase by TNF-α), leading to oxidative stress. [369-372] The production of superoxide anion and NO leads to the formation of peroxynitrite, reducing the bioavailability of NO to generate relaxation in the vessels. Moreover, pro-inflammatory cytokines can also generate production of oxidants and vice versa, creating a destructive cycle. [130,367,373] Arteries were incubated with 1400W to inhibit inducible nitric oxide synthase, thereby reducing inflammation and restoring vascular reactivity. Incubation with pegSOD, which breaks down superoxide anion, was hypothesized to reduce oxidative stress and restore vascular function. Furthermore, arteries were incubated with L-NAME to confirm the predominant role of NO in vasodilation and as the main pathway (NO-cyclic guanosine monophosphate) altered by *in utero* inflammation.

Following analysis of contractility in response to phenylephrine, no significant differences were observed, contrary to what was hypothesized. Despite the lack of differences, the LPS arteries showed weaker constrictive capacity compared to the Saline arteries. A possible explanation is that the generation of NO may increased after LPS exposure, thereby enhancing vasodilation. In support of these findings, Briones et al., observed a reduced ability of arteries to constrict following LPS exposure. [369] Mesenteric arteries isolated from spontaneously hypertensive rats had a significantly reduced contractile response to noradrenaline (0.1-30 μM) when incubated with LPS (10 μg ml^{-1}) with and without L-arginine (10 μM, substrate involved in NO synthesis). [369] The differences found may be due to increased protein expression of inducible nitric oxide synthase by LPS. [369] Moreover, these effects were observed in the normotensive Wistar Kyoto rats in the presence of both LPS and L-arginine. [369] LPS activates inducible nitric

oxide synthase via extracellular signal related kinase, janus kinase 2/signal transducer and activator of transcription 3, protein kinase C, and mitogen activated protein kinase, generating high concentrations of NO. [374-377]

NO has been shown to be produced by both endothelial nitric oxide synthase and inducible nitric oxide synthase induction following LPS exposure, in a time dependent manner, representing acute and delayed phases of septic shock. [370] A study by Takizawa et al. showed that LPS induced IL-1β (20 ng/ml) production and promoted vasodilation in rat aortas following exposure to phenylephrine (1 μM). [378] At 6 hours after IL-1β incubation, cyclic guanosine monophosphate was increased, which may in part explain the relaxation response observed, and was inhibited following incubation with L-NAME (100 μM). [378] At 24 hours, L-NAME and aminoguanidine (100 μM) were unable to prevent the relaxation response, however, incubation with tetraethylammonium, a non-selective K^+ channel inhibitor prevented the relaxation response. [378] The results suggest that LPS induced hyporesponsiveness is temporally regulated, where the NO-cyclic guanosine monophosphate pathway may be predominant during the early stages of inflammation, followed by activation of K^+ channels during prolonged inflammation.

Ding et al. identified that mice challenged with LPS had significant hypotension, increased inducible nitric oxide synthase and TNF-α expression, and suppressed endothelial nitric oxide synthase. [379] Using transgenic mice with a defect that inhibited activation of endothelium derived NF-κB prevented the alterations, suggesting that LPS mediates hypotension via activation of NF-κB. [379] Moreover, inhibition of Toll-like receptor 4 signaling (LPS receptor) was found to prevent hyporesponsiveness by hindering the activation of inducible nitric oxide

synthase and downstream inflammatory signaling. [380] Thus the pathway resulting in LPS-induced hyporesponsiveness may primarily involve activation of Toll-like receptor 4 and downstream NF-κB activation, NO-dependent and -independent increases in cyclic guanosine monophosphate, as well as temporally regulated K^+ channel opening. Thus, in addition to the temporal complexity of activated pathways, the type of arteries being examined is important, as different arteries may not respond the same way. [381]

It was expected that incubation with 1400W, an inducible nitric oxide synthase inhibitor, would restore LPS artery pressor responses to values similar to control. However, there was no significant effect of 1400W on the phenylephrine cumulative concentration response curve, EC_{50}, or E_{max}. This result was unexpected, given that LPS-mediated vasodilation is affiliated with activation of inducible nitric oxide synthase. [382] It is possible that at the time-point of evaluation, endothelial function was already restored. It was hypothesized that since inflammation and oxidative stress are involved in a continuous cycle, removal of one will fracture the cycle, leading to the dissipation of the other. However, no effects of pegSOD were found following EC_{50} and E_{max} analyses. Finally, L-NAME also did not have an effect on either EC_{50} or E_{max}. This is surprising as L-NAME is a nitric oxide synthase inhibitor, and decreasing the enzyme responsible for producing NO should enhance contractility of the vessels. A study by O'Brien et al. showed that the effects of L-NAME, 1400W, and ODQ (an inhibitor of the guanylyl cyclase) on LPS induced vascular dysfunction had a decrease in functional response over time (PE contractility responses were tested at 6, 20, and 46 hours). [383] By 46 hours, responses elicited by the inhibitors following the phenylephrine constriction curve were no longer as effective as earlier interventions. [383] To explore the role of different constricting

agonists, U46619 (thromboxane A_2 mimetic) was used to induce vasoconstriction, however no differences were detected, suggesting that thromboxane-meditated vasoconstriction is not involved in LPS induced endothelial dysfunction. [383] Inhibition of cycloxygenase-2, a downstream enzyme activated following exposure to LPS, did not reverse hyporeactivity. [383,384] The lack of responses by the LPS arteries to these inhibitors occurred at 20 and 46 hours; the vascular analyses in this study were conducted at 36 hours after the last LPS injection. Together, the results obtained suggest that LPS-induced vascular changes, at later time points, may no longer involve NO-dependent mechanisms, which may partially explain the lack of effects following inhibition. [383]

It was hypothesized that inflammation would cause endothelial dysfunction characterized by impaired relaxation. A relaxation curve induced by the endothelial dependent agonist methacholine showed completely relaxation of Saline arteries but not LPS arteries, but these differences were not statistically significant. The methacholine relaxation curve also revealed that LPS arteries were able to fully dilate at lower doses compared to Saline arteries; however, the arteries reconstricted at higher doses of methacholine, suggesting endothelial dysfunction. [385] The re-constriction results from impaired endothelial relaxation. [385] Methacholine would then activate muscarinic receptors found on vascular smooth muscle cells since endothelial cells are impaired, causing downstream activation of L-type calcium channels found on the former. [385] Following incubation of the arteries with the inhibitor 1400W or pegSOD no differences were observed between the groups in the dose response curve, the pEC_{50}, or maximal vasorelaxation. It is possible that other free radicals may be involved in the pathophysiology, such as H_2O_2, which has been implicated in endothelial dysfunction. [386] In support of this, treatment of pro-

oxidant generation with both vitamins C and E was shown to reduce oxidative stress and improve vasodilation in aortas isolated from the thoracic region. [131] Incubation with L-NAME did not result in differences in the pEC_{50} and E_{max}. Moreover, L-NAME did not completely abolish dilatation in Saline arteries, suggesting the possibility of compensatory mechanisms facilitating relaxation (i.e., prostacyclin and endothelium derived hyperpolarizing factor mediated vasodilation) or an effect of stage of pregnancy.

Inflammation-induced impaired endothelial functioning has been attributed to TNF-α induced reduction in endothelial nitric oxide synthase expression and phosphorylation of the enzyme, thereby preventing activation of protein kinase C. [379,387-389] Papapetropoulous et al. found that both LPS and IL-1β were capable of interfering with guanylyl cyclase signaling, thereby decreasing cyclic guanosine monophosphate levels. [390] TNF-α and cyclooxygenase-2 activation can also lead to increased production of superoxide anion promoting oxidative stress and reducing the bioavailability of NO. [391] Orshal et al. examined the effects of IL-6 (10 pg/ml – 10 ng/ml) on thoracic aortas isolated from virgin and pregnant (E20) Sprague Dawley rats and found that IL-6 enhanced contraction following exposure to phenylephrine and disrupted acetylcholine induced relaxation. [367] These effects were more pronounced in vessels from pregnant rats compared to non-pregnant rats. [367] In addition, incubation with L-NAME and an inhibitor of cyclic guanosine monophosphate enhanced contraction and prevented relaxation. [367] The arterial response to sodium nitroprusside induced relaxation and did not differ between controls and IL-6 treated vessels, suggesting that IL-6 may have an influence on the NO-cyclic guanosine monophosphate pathway. [367]Similar results were observed by Giardina et al.in TNF-α-treated aortic vessels isolated from virgin and pregnant Sprague Dawley rats (10-1000 pg/ml). [130]

No significant changes to blood flow or endothelial function were observed, which was contradictory to the hypothesis. The data suggest that this inflammatory model may not induce changes to blood flow nor cause significant endothelial damage. A possible explanation in the lack of impaired vascular response to both constrictor and dilatory agents may be due to the physiological state. During pregnancy, an increase in blood flow to the uterus supports the developing placenta and fetus. To accommodate this, the vasodilatory properties of the uterine vasculature are increased. [392] This enhancement of vasodilation may overcome and mask any effects caused by the low-dose exposure to LPS since the vascular responses were evaluated a day prior to delivery. The vascular responses obtained in this study may also be influenced by cytokine imbalances that occur during maternal inflammation. The temporal profile of inflammation may influence the outcome of the vascular responses; short-term exposure to cytokines may enhance vasoconstriction whereas prolonged exposure results in hyporesponsiveness. [393] Cytokines have been suggested to exert activation of cycloxygenase-2 and/or pro-endothelin-1, leading to downstream activation of thromboxane A_2 and endothelin-1, respectively, resulting in vasoconstriction. [393] Alternatively, cytokines can activate cycloxygenase-2 and inducible nitric oxide synthase, leading to increased production of prostacyclin and NO respectively, and resulting in vasodilation. [393] However, in the current study, rather than using cytokines, LPS was administered to pregnant rats and arteries were excised and tested for their vascular responses 2 days after the final injection. Several studies apply cytokines or LPS directly into the baths containing the arterial segments and evaluating their functional capacity within hours, thus, the effects may be more robust. Furthermore, alterations to pro-inflammatory cytokine mRNA expression in the placentas no longer evident by

E22, suggesting that the inflammatory process was no longer present and vessel function may have been restored by the time of assessment. Additional limitations also include a small sample size (*n*=4 dams) and lack of investigation into alternative mechanisms involved (i.e., prostacyclin, thromboxane A_2, and endothelin-1). Alternatively, the data point selected may have been too late to detect changes since blood flow analyses were performed a day after the final injection and artery function was assessed two days after the final injection. The expression of endothelial nitric oxide synthase and inducible nitric oxide synthase was not evaluated, therefore the role of LPS on these enzymes are speculations. [370,394] Furthermore, the presence of cytokines and pro-oxidants were not evaluated, therefore further experiments into the role of *in utero* inflammation is required.

Although we were not able to confirm endothelial dysfunction as a result of LPS in this study, future studies may improve on the limitations identified in the current study and measure the possible therapeutic effects of BrSp dietary supplementation. BrSp are effective at ameliorating both oxidative stress and inflammation, and thus, may be a powerful intervention to protect against endothelial dysfunction following maternal inflammation. [230,231] Studies have shown that inhibition of either inflammatory or oxidative stress pathways is effective in restoring vascular function. [372,395] BrSp are a potent endogenous phase II enzyme inducer capable of eliminating oxidative stress whether caused by superoxide anion or hydrogen peroxide. Furthermore, BrSp is also effective in reducing the inflammatory response. Thus, BrSp poses as a potential therapeutic intervention to restore vascular functioning. [230] Furthermore, caution should be used when interpreting and comparing vascular studies, as differences have been observed between the types of vessels (i.e., systemic, resistance, pulmonary), endotoxin model

and timing of experimentation, strain and species of the model, sexes, virgin vs. pregnant animal models, agonists used to induce constriction and vasodilation, etc. [130,367,381,383,396]

5.4. CONCLUSIONS

Following *in utero* inflammation, uterine and umbilical blood flow was not adversely affected at the time points evaluated. LPS arteries did not display enhanced constriction or impaired relaxation. Furthermore, the EC_{50} and E_{max} values were not significantly different. Maternal inflammation did cause a placental inflammatory response characterized by an upregulation of all four cytokines measured (IL-1β, TNF-α, IL-6, and IL-10) on E19 but were no longer significantly different on E22. Placental inflammation did not have an immediate effect on proinflammatory cytokines in the developing brain at E19. But on E22, there was an increased expression of fetal brain IL-1β. Alterations to cytokine levels induced a decreased expression of NGF on E22 in the fetal brain. Although BrSp were able to prevent IL-1β expression, their beneficial effects were not sufficient to prevent changes in NGF mRNA expression. The role of oxidative stress in this model is limited as increases in MMP-2 activity and changes to the reduced/oxidized glutathione ratio were not observed in newborn brains. Delays in developmental reflexes, and behavioural anomalies were present in LPS offspring, and these deficits were not evident in most reflexes and behavioural outcomes in the LPS + BrSp group. Furthermore, BrSp dietary supplementation was able to partially protect the brain against white matter injury by preventing the loss of 2',3'cyclic nucleotide 3'-phosphodiesterase.

Our model reflects a subtle model of CP induced by fetal inflammation, whereby partial protection to developmental reflexes and maturation of oligodendrocytes were conveyed by

consumption of BrSp during late pregnancy and the pre-weaning periods. This subtle model is more reflective of the largest segment of the CP population. [397] Therefore, it is important identify therapeutic interventions for infants at risk. BrSp may be a safe and effective intervention that can be administered to all pregnant women. [241] Our studies and others' have shown that BrSp consumption during pregnancy is protective for both mother and fetus susceptible to hypertension, placental insufficiency, and inflammation. [230,231,243,398] Furthermore, prenatal inflammation may be a common predisposing factor responsible for neurodevelopmental disorders, neuropsychiatric disorders, and neurodegenerative diseases, and the protective properties of BrSp may be extended to these disorders. [49,51,399] Our study highlights the potential beneficial effects of BrSp consumption during late gestation for improving cytokine imbalances, maturation of oligodendrocytes, developmental delay, and behavioural anomalies.

6. FUTURE DIRECTIONS

A power and sample size analyses was performed and the planned experiments will increase the sample size to *n*=8 for mRNA and maternal vascular analyses. Since the current data reflects *n*=4, interpretations are subject to change. Furthermore, we aim to characterize the neuroprotective effects of BrSp on Nrf2, the main transcription factor that is affected by SFN exposure and influences several protective pathways including phase II and antioxidant enzymes, growth factors, cell signaling pathways, and inflammation.

In this study, offspring were tested up to PD21, reflecting a young human infant (approximately 1-2 years). It is crucial that the behaviour resulting from this inflammatory model at adolescent (13-16 years) and adult ages (20s) are evaluated (PD35 and PD80 respectively). Testing will include the Morris water maze, single pellet, tapered beam, elevated plus maze, open field, and novel object recognition. These tests will determine whether our model of maternal inflammation results in behavioural abnormalities into adolescence and adulthood. Furthermore, whether BrSp dietary supplementation during late pregnancy and the preweaning period is sufficient to incur neuroprotection into adulthood will be assessed.

Following behaviour analyses, changes in the brain at PD35 and PD80 will be assessed. Olig2 and myelin constituents will be measured to determine whether white matter injury is present in the adult period and if the protective properties of BrSp are effective in preventing myelination abnormalities at this stage. CD68 and glial fibrillary acidic protein expression will be measured to determine the inflammatory state of the adult brain. Neurotransmitter production

and synaptogenesis alterations will also be assessed since neuronal dysmaturation may be a pathomechanism involved with the FIR, leading to future cognitive and behavioural deficits. Golgi-Cox staining on neurons will also be performed, to determine whether neurons are maturing and properly developing properly.

Using microarray techniques, studies have identified pathways that are influenced by both inflammation and SFN. Oskvig et al. found that 4 hours after the induction of maternal inflammation, the top genes upregulated were associated with hypoxia, cell stress, cell death, and immunological mediators. [400] The top downregulated genes were involved with interneuron migration, axon guidance, axon morphology, *gamma*-aminobutyric acid migration, synaptic scaffolding, brain development, and transcriptional regulation. [400] Kraft et al. found that in an *in vitro* model of oxidative stress, the antioxidant response element activation, the downstream target of SFN, afforded neuroprotection against the insult via activation of astrocytic and neuronal genes including detoxification enzyme, calcium and cell adhesion signaling pathways, synaptic signaling, inflammation, and many others. [239] Employing microarray analyses in pending studies will permit the identification of pathways altered in the brain in response to inflammation and identify alternative protective pathways induced by SFN.

Overall, the goal of future studies is to identify the neuroprotective properties of BrSp consumption during late gestation and the pre-weaning period as a therapeutic intervention to protect the developing brain against fetal inflammation. This could ultimately reduce the incidence of CP, among many other neurodevelopmental and neuropsychiatric disorders induced by the FIR.

7. CONCLUDING SUMMARY

CP is comprised of a heterogeneous population both physically and mechanistically, thus it is important to elucidate all possible mechanisms of injury and to identify safe and effective protective interventions. The model used in these studies mimics an inflammatory insult during the early periods of fetal vulnerability (22-26 weeks gestation) and leads to a mild-moderate brain injury sufficient to induce dysmaturation of neurons and glial cells, similar to the mild spectrum of CP. Therapeutic intervention with BrSp, in this model of FIR and CP, prevented the aberrant outcome. It seems reasonable, therefore, to consider BrSp consumption during pregnancy and pre-weaning as a novel therapeutic intervention against the neurodevelopmental disorders (i.e., CP, autism) induced by antepartum insults caused by fetal inflammation.

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