University of Alberta

Broccoli Sprout Supplementation during Placental Insufficiency Confers Structural and Functional Neuroprotection to the Fetal Rat

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Master of Science

Centre for Neuroscience

Spring 2010
Edmonton, Alberta

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Dedication

For the many beloved people in my life who have supported my commitment to this project and achievement of my Graduate Degree.

I want to express sincere gratitude to my Mother and Father for instilling in me a love of learning and an appreciation for education of all things including science, literature, religion, art and music. They also taught me to reach for the stars, to challenge myself and always encouraged that anything could be accomplished with hard work and perseverance.

To Dig, hsoJ, DD and Pudd, the other musketeers. We are not unlike the intricate workings of the central nervous system – much greater than the sum of our parts. All for one and one for all!

To Kelly, who covered me when my ammunition ran out and carried me when I thought I would give up.

To Tobin, Kassidy, David and Dallin, who are my inspiration. Mommy thanks you for being so independent and often helping yourselves. Although it was hard to understand what I was doing, you did your best to be patient. I want you all to know that you four are by far the greatest accomplishment of my life.

Finally, to my Starla, who never stopped believing in me even when I didn't, who partnered with me in raising my four children (and 4 of her own) for 10 years while I studied, who was always there when I needed **anything**, who laughed, cried and screamed with me, who listened to everything I ever had to say (which is A LOT!) and who deserves an honorary degree for being the one behind the scenes. Without you, Starla, none of this dream would have been possible. I will be forever in your debt.

Abstract

Background: Perinatal ischemic brain injury leads to developmental disability (DD), which accounts for 30% of disabilities in children. Antepartum risk, or risk occurring prior to birth occurs in more than 90% of cases. This study investigated whether maternal ingestion of a natural health product (broccoli sprouts) would provide neuroprotection in an intrauterine model of HI.

Methods: Intrauterine ischemia was induced by bilateral uterine artery ligation (BUAL) on E20 of gestation. Rats were fed broccoli sprouts (200 mg) from E15 until postnatal day 14 (PD14). Rat pups underwent neurobehavioural testing from birth to PD21 and were then sacrificed for neuropathologic assessment on PD21.

Results: BUAL ligation resulted in growth restriction (IUGR) of the fetuses, which persisted throughout the study ($p < 0.001$). Reflex testing indicated IUGR pups were developmentally delayed compared to controls ($p < 0.001$). Open field testing on PD21 indicated hyperactivity in IUGR animals compared to controls ($p < 0.001$). Histological assessment showed a reduction in pyramidal cells in CA1 and CA3 of IUGR hippocampi and in myelin basic protein (MBP) immunohistochemistry signal. Broccoli sprout supplementation improved some reflex and behavioural measures, increased cell counts in CA1 and CA3 as well as MBP signal in growth restricted animals.

Conclusions: Supplementation with broccoli sprouts during the last trimester of gestation and the first 2 weeks of life in the rat lessened the effects of chronic intra-uterine ischemia. These findings suggest a novel approach to the prevention of DD associated with perinatal HI.

Acknowledgements

I would like to acknowledge the following individuals for their contribution to this research project and/or support of my endeavours: Jerome Yager, for giving me a vision; Kathryn Todd, for inspiring my interest in Neuroscience; Frederick Colbourne, for teaching me about scientific integrity, scepticism and attention to detail; Ed Armstrong, for knowing everything I didn't and helping with every step of my project; Bernard Juurlink, Marc Del Bigio, Paul Talalay, Greg Parks, Sandy Davidge and Jude Morton for their support, instruction and contributions.

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Abbreviations

ANOVA – Analysis of Variance

BUAL – bilateral uterine artery ligation

CP – Cerebral Palsy

DD – Developmental Disabilities

ELISA – enzyme-linked immunosorbent assay

GFAP – glial fibrillary acidic protein

GPx – glutathione peroxidase

GSH – reduced glutathione

GSSG – oxidized glutathione

H&E – Haematoxylin and Eosin

HI – Hypoxia-Ischemia

HIE – Hypoxic-Ischemic Encephalopathy

IUGR – Intra-uterine Growth Restriction

MBP – myelin basic protein

NMDAr – N-methyl D-aspartate receptors

PD – Postnatal Day

ROS – Reactive Oxygen Species

SD – standard deviation

SOD – superoxide dismutase

Introduction

Perinatal hypoxic-ischemic (HI) brain injury is a major cause of neonatal hypoxic ischemic encephalopathy (HIE) resulting in neurodevelopmental disabilities inclusive of mental retardation and cerebral palsy (CP) (Ferriero, 2004). Birth prevalence of HIE ranges from 2 to 6 of every 1000 term births and increases dramatically, up to 50%, with the inclusion of preterm births and antepartum (before birth) risk factors (Miller et al., 2005; Volpe, 2009). Placental insufficiency is one such risk factor, which results in intra uterine growth restriction (IUGR) of the fetus, reported to increase the probability of HIE by 40-fold (Badawi, 1998). The perinatal brain has immature systems of cerebral vasculature and metabolism (cellular defense against reactive oxygen species and environmental toxins) and specialized systems of development, making it uniquely vulnerable to adverse events or interruptions during gestation (Olney, 2002; Back, 2006). Advances in health research have improved survivability of these infants, but simultaneously increased the prevalence of associated developmental disabilities (Back, 2006). Unfortunately, identifying the at-risk fetus and targeting optimum time windows for therapy remains a challenge in current clinical settings. In addition, virtually all therapies developed to target HIE have not translated into clinical trials for the newborn as numerous pharmacological agents have proven neurotoxic to the developing brain (Olney et al., 2004; Black et al., 2008). In this regard, increasing survival of preterm/low birth weight infants raises concern, as the rates of poor neurodevelopmental outcome, potential costs to health care and educational systems, as well as the emotional impact on the affected families, have increased over the years

(Boyle et al., 1994; Leitner et al., 2007a).

Perinatal (HI) brain injury broadly describes a range of possible insults and outcomes for affected individuals. According to the National Foundation for Brain Research (USA), a spectrum of disorders related to prematurity and HI account for 27% of all childhood disability. Severely affected children present with difficulties like CP, mental retardation or epilepsy while milder, but commonly reported problems include learning disorders, hearing and/or vision impairments, speech deficits, growth delays, and emotional/behavioural abnormalities (Boyle et al., 1994; Msall et al., 2003; Leitner et al., 2007a). A recent study evaluating long-term neurodevelopmental outcome (15-19 years) in children born with moderate newborn encephalopathy found 81% had some degree of cognitive dysfunction with or without cerebral palsy, indicating the majority of infants exposed to an hypoxic-ischemic insult will likely face lifelong challenges (Lindstrom et al., 2008).

Intra-uterine growth restriction resulting from placental insufficiency has been found to be independently associated with developmental disability (DD) and brain damage in both animal and clinical studies. Olivier et al., (2005) used the unilateral uterine artery ligation (UUAL) rat model of growth restriction and found significant white matter injury, accompanying macrophage infiltration and astrogliosis as well as a myelination deficiency that persisted to adulthood (Olivier et al., 2005). A recent clinical study revealed a suboptimal neurodevelopmental outcome in 28.6% of IUGR children at 9-10 years of age (Leitner et al., 2007b). Imaging studies have also reported evidence of altered brain development and associated psychiatric symptoms in adolescents (14-15) who were born with IUGR (Indredavik et al., 2005; Skranes et al., 2008). These

data reflect outcomes following a relatively mild insult to the perinatal brain. Combinations of factors (e.g. IUGR+prematurity) are more common and pose a risk of more severe injury. However, this research indicates the importance and implications of a single antepartum risk factor for perinatal brain injury.

Historically it was thought that brain injury in the newborn could be primarily attributed to asphyxia at the time of birth (*intrapartum*). We now know HI can occur anytime during the *perinatal period*, a continuum ranging from approximately 20 weeks gestation until one month after birth (Yager et al., 2009). In fact, recent evidence suggests that *antepartum* risk (e.g. IUGR) is involved in up to 94% of cases. That is to say, many causal pathways that culminate in birth asphyxia originate in the antepartum period (Badawi et al., 1998; Hankins and Speer, 2003; Keogh and Badawi, 2006). These results are supported by related research showing between 70-90% of predisposing factors for DD begin before birth (Jacobsson and Hagberg, 2004; Low, 2004). The exact contribution of antepartum events is unknown and research has not yet provided sure methods of identification. Unfortunately, high-risk pregnancies often proceed undetected by health professionals.

What we do know is that the perinatal period is a critical period for brain growth and maturation. The human brain growth spurt, originally described by Dobbing (1974), is the period when the brain is experiencing its most rapid stage of development (Dobbing, 1974). It has been well established that due to the rapid development of the perinatal brain, different patterns of injury and specific regions of vulnerability emerge depending on gestational age at the time of the insult. Evidence of predominantly white matter injury in preterm infants with HIE in contrast to a predominantly deep basal nuclei

(gray matter) pattern of damage in term newborns with HIE indicates the importance of timing in the underlying pathogenesis of injury (Ferriero, 2004; Miller, 2007). Much research has been done and is underway to better understand differences in mechanisms of perinatal brain injury depending on timing, however this knowledge is insufficient to make long-term predictions and design therapeutic strategies because as many as 30% of afflicted infants with moderate HIE recover and have normal outcomes (Volpe, 2001c). Long-term neurodevelopmental outcome depends more on the severity of the injury. Timing is one important factor, but the duration of the insult, gestational age at birth, any antepartum risk factors, genetic predispositions as well as prenatal and post-partum care all play a role in determining long-term outcome (Badawi et al., 1998; Miller, 2007).

Susceptibility of the immature brain to HI can generally be attributed to immature systems of vascularisation, metabolism and cellular defense as well as specialized systems for the development of synaptic neurotransmission. For example, during the critical window for development of perinatal white matter injury (PWMI), long and short penetrating arteries in the sub cortical and periventricular areas are not fully developed creating “vascular border zones and end zones”, which are particularly vulnerable to an ischemic episode (Volpe, 2001b). The pathogenesis of post-ischemic injury involves a cascade of biochemical cellular events with two major resultant downstream mechanisms, 1) over production of reactive oxygen species (ROS), or oxidative stress and 2) excitotoxicity.

The vulnerability of the developing brain to ROS can be at least partially explained by an immature metabolism. Before term gestation, there is a lack of

efficiency in defensive compensatory mechanisms to combat an overproduction of ROS. Oxidative stress refers to the overproduction of strong oxidants like superoxide anion (O^{2-}), which reacts with NO to form peroxynitrite, initiating lipid peroxidation (Blomgren and Hagberg, 2006). Central to cellular defence against ROS are three endogenous enzymes, superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) (Warner et al., 2004). Immature functioning of these enzymes, particularly of white matter, is thought to be a factor in susceptibility of the premature brain to oxidative injury. For example, while catalase and GPx reach adult levels in developing white matter by 30 weeks gestation, SOD lags until term (Folkerth, 2006). Oligodendrocytes (OLs) are the white matter glial cells of the brain. The maturation-dependant vulnerability of OLs to oxidative stress has been delineated by Back et al., (1998, 2001 and 2002) and is suspected to be the underlying cause of perinatal white matter injury (Back et al., 2007).

Excitotoxic pathways also vary depending on developmental stage of the brain. Classically defined, over activation of glutamate at N-methyl D-aspartate receptors (NMDAr) leads to excitotoxic cell death. In the immature brain, under activation of glutamate at these receptors also has deleterious consequences, resulting in apoptosis (programmed cell death or cell suicide) (Olney, 2003). Immature neurons have a greater propensity for apoptosis than mature neurons due to natural pruning of unnecessary cells or deletion of cells unsuccessful in making connections (Bayly et al., 2006). Development of the NMDAr system has not been fully elucidated, but it is considered a major signalling pathway for neuronal migration and synaptogenesis, as well as maturational changes in cell metabolism and gene expression (Haberny et al.,

2002). Variation in combinations of NMDAr subunit expression between the adult and newborn brain is considered to account for differences in function and response to agonistic/antagonistic agents.

Inherent vulnerabilities unique to the brain growth spurt and related to NMDAr function have limited the potential for treatment development for newborn brain injury. Therapies such as NMDA antagonists, developed for adults with ischemic brain injury, as well as conventional therapies like caffeine for apnea of prematurity and common anti-convulsant medications have proven harmful to the developing brain (Ikonomidou et al., 1999; Olney et al., 2004; Black et al., 2008). Thus, medical professionals are faced with a conundrum. Conventional therapies can potentially do more harm than good, and there is not yet a sure method for identifying risk. Moreover, medical technology cannot yet reliably determine the timing of injury, or its duration, making the institution of therapeutic intervention in a timely beneficial fashion, impossible. There is therefore, a critical need for the development of neuroprotective strategies that are safe for both mother and fetus and that will work in a preventative fashion during pregnancy and throughout the neonatal period.

Natural health products provide an interesting and promising alternative to conventional medicines for protection from oxidative-stress induced brain injury following perinatal HI. Recent discoveries in the field of chemoprevention are pointing to the family of *Cruciferae* (cruciferous) vegetables, particularly of the genus *Brassica* to be particularly effective in reducing tumor formation through an antioxidant mechanism (Fahey and Talalay, 1999). Broccoli sprouts have been shown to contain the highest concentration of the precursor, glucoraphanin, a glucosinolate which is hydrolyzed

during metabolism to the isothiocyanate, sulforaphane, a phase-II enzyme inducer and therefore, powerful antioxidant (Fahey et al., 1997). Phase-II enzyme induction provides indirect antioxidant action by enhancing the synthesis of glutathione, the most important endogenous free-radical scavenger of cells (Juurlink, 2001). While conventional antioxidants such as Vitamin E, act directly to reduce reactive oxygen species (ROS), phase II enzyme induction indirectly boosts the intrinsic cellular anti-oxidant response. Sulforaphane has gained recognition as a chemoprotective compound, but several studies have also shown sulforaphane to reduce oxidative stress, hypertension and inflammation in the cardiovascular system of spontaneously hypertensive rats (SHR) (Fahey et al., 1997; Juurlink, 2001; Wu and Juurlink, 2001; Wu et al., 2004). In this regard, sulforaphane may serve to diminish the adverse effects of cellular oxidative stress, which is one of the major contributing mechanisms of HI brain injury in the newborn (Yager and Thornhill, 1997; Volpe, 2001a).

Studies to date, investigating the role of neuroprotective agents in the newborn have traditionally utilized the post-natal rat model (Yager and Ashwal, 2009). Given that the majority of risk occurs during the ante-partum period, a model of intra uterine injury was necessary. In this study I therefore used a rodent model of utero-placental insufficiency to induce chronic intra-uterine ischemia and perinatal brain injury. Placental insufficient models have been previously shown to result in growth restriction of the fetuses as well as cause alterations in brain development and cell damage (Olivier et al., 2005). Hallmark features of clinical placental insufficient IUGR, such as very low birth weight and asymmetric head to body ratio, are closely mimicked in the rodent model, suggestive of a fair extrapolation of findings.

The following Hypotheses were formulated: 1) Chronic Placental Insufficiency induced by BUAL will produce a model of IUGR and developmental delay that mimics the human newborn; 2) Supplementation of the maternal diet with broccoli sprouts, during gestation and the early newborn period, will reduce adverse neurodevelopmental effects of a perinatal hypoxic-ischemic insult; 3) Improvement in neuropathologic outcome will coincide with functional improvement.

Methods and Materials

Animals

Female (virgin) Long-Evans rats, 12-16 weeks old and weighing 300-400g (Charles River Laboratories) were used in this study (n = 17). Animals were housed in the Health Sciences Laboratory Animal facility (HSLAS) at the University of Alberta. Following a 5 day handling and acclimatization period, animals were timed-pregnant whereby a vaginal lavage containing sperm denoted day one of gestation (E1). Pregnant dams were then assigned to one of four groups in no particular order but as available depending on breeding success and availability of HSLAS surgery suite space. Experimental groups included: *1) Sham operated (SHAM); 2) Sham operated with broccoli sprout supplement (SHAM+B); 3) Bilateral Uterine Artery Ligation operated (BUAL); 4) BUAL operated with broccoli sprout supplement (BUAL+B).*

Rat pups were born spontaneously, and reared with their dams in conventional housing. Litters were culled to 8 where more than eight pups were born. From 3 - 6 different litters of pups were used to represent each of the four experimental groups throughout the study (n = 88 pups). All animals were maintained on a 12-hour light/dark schedule and received food and water *ad libitum* throughout the study. All procedures have been approved by the Health Sciences Animal Care and Use Committee at the University of Alberta and are in accordance with the Canadian Council on Animal Care guidelines.

Surgical Procedure

Bilateral Uterine Artery Ligation (BUAL), adapted from Wigglesworth's original model of fetal growth restriction, was performed on day 20 of gestation (E20) in order to induce chronic placental insufficiency (Wigglesworth, 1974; Lane et al., 1998). This stage of brain development for the fetal rat is thought to compare to human brain development at approximately 22-26 weeks gestational age or the beginning of the third trimester (Rice and Barone, 2000; Yager, 2004).

Pregnant rats were anesthetised with 4% isoflurane (approximately 2% maintenance) in medical air (21% oxygen) and received a vertical, low midline abdominal incision, approximately 2-3 cm long. Both uterine arteries, proximal to the uterine bifurcation, were permanently ligated with 4-0 Vicryl coated suture (Ethicon Inc., Somerville, NJ, USA). After suturing the muscular layer with the Vicryl, 0.05ml of bupivicaine (Sensorcaine by AstraZeneca Can Inc., Ont., Can.) was administered in a drop wise fashion for the purpose of analgesia and finally the skin layer was closed with 5-0 silk suture (Angiotech, Surgical Specialties Corp., Reading, PA, USA). Following surgery, the animals were closely monitored over a 4-6 hour period to ensure a full recovery, with no complications. Sham operated rats underwent identical anaesthetic and surgical procedures with the exception of uterine artery ligation. All surgical procedures were performed according to aseptic technique.

Intrauterine Growth Restriction

Intrauterine growth restriction (IUGR) is clinically defined as a birth weight less than the 10^{th} percentile for gestational age. For this study, the definition previously

described by Olivier et al., 2005, was used, which is a birth weight ≤ 2 SD below the mean (Chang et al., 1992; Resnik, 2002). Preliminarily, birth weights were collected from four naïve litters (n = 56) to ascertain the mean birth weight for our breeding colony and define the criteria for IUGR. (Mean = 6.28 g, SD = 0.38 g, IUGR ≤ 5.52 g).

Bilateral uterine artery ligation is a placental insufficiency model of growth restriction, resulting in lower birth weight and a “brain-sparing” phenomenon of the fetuses. The latter refers to ‘asymmetrical’ growth restriction, which predominantly occurs as a result of placental insufficiency and generally takes effect in the third trimester when the majority (2/3) of fetal growth is completed (Campbell and Thoms, 1977; Rosenberg, 2008). Individual litters of SHAM (n = 10 pups) and BUAL (n = 14 pups) were used to measure weight and head circumference at birth and then the calculation for cephalization index (CI) as previously described by Bassan et al., (2005), (CI = head circumference/birth weight) was done to assess growth symmetry of the pups and establish clinical significance of the BUAL model.

Long Evans rats have a typical gestation of 23 days. The delivery date for the purposes of this study will be referred to as postnatal day 1 (PD1). On the day of delivery, newborn rat pups were counted, weighed, sexed and returned to their dam. Only litters of at least 5 pups were included in the study and only those pups weighing equal to or less than 5.52 g from BUAL dams were considered to be IUGR and included in the study. Following initial measurements, pups were allowed to nurture with their dam for approximately 48 hours to ensure a healthy attachment between mother and young. Beginning on postnatal day 3 (PD3) rat pups were weighed daily to monitor growth and confirm adequate nutrition until PD14 and then finally on PD21. Historically,

in the Yager Laboratory, healthy rat pups gain approximately 10% of their body weight per day and a drop of 20% below their expected age weight is considered failure to thrive. Any pup with a consistent weight loss pattern resulting in failure to thrive was euthanized by induction with isoflurane (5%) in 30% oxygen and balanced nitrogen and decapitation.

Broccoli Sprout Preparation and Supplementation

The appropriate groups received broccoli sprouts as a supplement to their regular rat chow beginning on E15 of gestation (beginning of the third trimester) until PD14 of the pups. Broccoli sprouts were provided for this study from the Juurlink Laboratory at the University of Saskatchewan and were prepared as previously described (Noyan-Ashraf et al., 2005). Calabrese cultivar broccoli seeds (Mumm's seeds and sprouting, Shellbrook, SK, Canada) were sprouted according to supplier's instructions. Four-day-old sprouts were air dried for 7 days and stored at room temperature. This cultivar was chosen based on previous research that shows glucoraphanin is the predominant glucosinolate and anti-nutritive glucosinolate content is low (Wu et al., 2004). Rats received 200 mg of dried broccoli sprouts per day as a supplement to their regular rat chow. From E15 and continuing until PD14, a small ceramic dish containing the sprouts was placed in the home cage. Previous research has shown 200 mg of dried broccoli sprouts contained phase-II protein inducer activity that was equivalent to 5.5 μmoles of sulforaphane (Wu et al., 2004). Previously in the Yager Laboratory it was shown that 200 mg of dried broccoli sprouts fed to pregnant dams provides 31.9 ± 4.64 pmol/mg of active enzyme to the fetuses, comparable to an

intraperitoneal injection of 500 µg of sulforaphane, which provided 27.0 ± 8.31 pmol/mg of protein (*manuscript in progress*).

Evaluation of Neurobehavioral Development and Maturation

Reflex and behavioural testing began on PD3 and continued daily through to PD21 to assess the emergence of reflexes, maturation and other sensorimotor behaviours. Following transport from the animal facility, rats were allowed to acclimatize to the testing environment for approximately one hour. The rat pups were removed from their home cage for testing every afternoon. The testing environment was a separate room within the laboratory, which is quiet and void of white noise. Newborn rats have an immature thermoregulatory system and are highly susceptible to hypothermia, which could have a significant effect on their ability to perform the required tasks (Kreider and Blumberg, 2005). To avoid temperature related behavioural changes during testing, newborn rats were tested in an incubator maintained at 34.5ºC where possible or under a warm lamp (31ºC) for tests that could not be performed inside the incubator, e.g. righting, which was a video-monitored test.

Postnatal day of attainment for each individual reflex for each pup was recorded, except for the righting reflex where the duration of time to accomplish the task was recorded. The following is a description of the reflex tests that were used, which were adapted from the original work of Fox (1965) and Lubics et al., (2005).

1. Righting Reflex

The animal is positioned gently on its back on a smooth surface and will immediately turn over to rest in a normal prone position with all four feet on the ground. This test

was video-monitored and time to perform body righting was recorded from PD3-7. Maximum time given to complete the task was 15 seconds.

2. Grasp Reflex

The palm of the fore and hind feet are stroked with a blunt instrument and the limb flexes to grasp the instrument. Beginning on PD3, the day each pup could grasp the instrument with both, fore or hind limbs, was the postnatal day of attainment for that reflex.

3. Hind limb Placing Response

While suspending the pup gently, the dorsal side of one hind paw is touched to the edge of a flat surface causing the foot to be raised and be placed on the flat surface. Beginning on PD4, the day each pup performed the placing task with both hind limbs was the day of attainment.

4. Cliff Aversion

When the rat pup is placed at the edge of a flat surface (cliff or table top) with forepaws and head over the edge, it will turn immediately and crawl away from the cliff edge. Beginning on PD4, this test was scored 0, 1 or 2 according to each pup's ability (0, when the rat pup completely disregards the cliff; 1, when the pup attempts to struggle, but shows hesitation or does not fully avert the cliff edge; 2, when the pup immediately and confidently performs the aversion). The postnatal day each pup scored a 2 was the day of attainment.

5. Gait

Beginning on PD6, pups were individually placed in the center of a white paper circle (15 cm in diameter) and timed with a stopwatch. The day they began to move off the

circle with both forelimbs within 30s, was recorded. Longer than 30s to perform this task was considered a negative result.

6. Acceleration Righting

The animal is suspended upside-down by fore and hind limbs and suddenly released falling about 12 inches into a sturdy box lined with a thick piece of foam to cushion the fall. The successful animal will turn and right itself mid-air in order to land on all fours. This test began on PD12 and was video-monitored in order to view the landing of the animals in slow motion. A partial attempt to right was given a score of 1 and complete acceleration righting was given a score of 2. The day each pup scored a 2 was considered the day of attainment.

Signs of Maturation

1. Auditory Startle/Eye Opening

A loud sharp noise will cause a rat to startle when auditory processing emerges. The first day a clapping sound produced an observable startle response, defined as a whole body twitch, was considered the day of attainment (Beard et al., 2006). The first postnatal day both eyes were opened was recorded for eye opening maturation. Eyes were considered to be open even if only a small opening was visible.

2. Posture

Rat pups crawl using mainly their forelimbs until about PD10 when they begin to use a quadruped stance. Beginning on PD12, rat pups were individually observed daily for stance and limb placement. The first day the pups could maintain a stance where all four limbs were held comfortably beneath their body both during rest and locomotion

was considered attainment of a mature posture for the purposes of this study (Fox, 1965; Bekoff and Trainer, 1979).

Open Field Behaviour

On PD21, animals were video-monitored for five minutes in an open field to observe various motor and exploratory behaviours. After acclimatization (at least one hour) to the testing environment, pups were placed in a 45 X 45 X 30 cm Plexiglas box. The floor of the box is divided into 4 X 4 cm areas. Each animal was placed in the center of the box facing the same direction. Recordings were evaluated by a blind observer. Parameters measured include: ambulation (number of squares crossed), rearing (both forepaws leave the ground), grooming behaviour, head lifting (lifting the head and neck in an exploratory manner, similar to rearing, but forepaws do not leave the ground) and defecation (Figure 1) (Lubics et al., 2005; Kiss et al., 2007).

Histopathology

On PD21 the rat pups were euthanized with 5% isoflurane in 30% oxygen and balanced nitrogen. They were then decapitated and following quick removal from the skull, the brains were flash frozen in iso-pentane and stored at -70 C˚. Coronal brain sections (10 µm) were cut on a cryostat (Leica, cryocut 1800) at -17 C˚ at the level of the anterior commissure (approximately 1.6 mm anterior to bregma), mamillary bodies (1 mm posterior to bregma), and through the midbrain (2.5 mm posterior to bregma) (Sherwood and Timiras, 1970). Sections were then mounted on slides and stored at -

20 C˚. Tissue sections were used for immunohistochemistry or stained with haematoxylin and eosin (H&E) for cell counting and cortical measurements.

Hemisphere, Cortical & Hippocampal Measurements

Basic measures of hemisphere width, cortical thickness and hippocampal lengths of SHAM and IUGR animals were measured to account for any differences in brain size that may be attributable to the nature of growth restriction in general, which could confound further histological evaluation. H&E stained tissue sections (2.5 mm posterior to bregma) were used for hippocampal measurements, anterior and posterior planes were used for hemisphere measurements and all three planes (H&E) were used for cortical measurements. Hemisphere width was measured from the most lateral aspect of the section to the midline alternating sides (left/right) per section and averaging across anterior and posterior planes. Cortical thickness measurements were done similar to Kolb and Cioe (2001) where three cortical measurements (dorsal, lateral and inferior) were taken on each of our three section planes and averaged for each measure at each plane (Figure 2) (Kolb and Cioe, 2001). The hippocampal length was assessed by measuring the distance across the hippocampus from the most lateral aspect of CA3 to the midline. Measurements were taken with the Image J computer program, version 1.36b (developed at the NIH, U.S.A., available at http://rsb.info.nih.gov/nih-image/) on images digitized using a stereoscope with a Spot Flex CCD camera and Spot Software.

Hippocampal Cell Counting

H&E stained tissue sections (2.5 mm posterior to bregma) were used to count

hippocampal pyramidal neurons. Loss of cells was assessed by counting the number of remaining healthy looking pyramidal neurons, not eosinophilic and having a distinct nucleus, in the CA1 and CA3 sectors of the hippocampus. The method used to count these areas was similar to that used by Colbourne and Corbett (1995). Briefly, under a microscope (400X magnification), medial and middle sectors of CA1 (0.5 mm long) in both hemispheres were counted and averaged. The CA3 count was determined by centering the microscope field of view (400X magnification) on the most lateral portion of the hippocampus (where pyramidal cells make a sharp turn medially) and all viable cells in this view were counted (Colbourne and Corbett, 1995) (Figure 3).

Immunohistochemistry (GFAP & MBP)

Brain tissues sections were stained with glial fibrillary acidic protein (GFAP) (Z 0334; DakoCytomation, Glostrup, Denmark), to investigate any astrocytic response to injury and myelin basic protein (MBP) (SMI 94; Cedarlane Laboratories Ltd., Ont., Canada) to examine development of myelinated white matter fibres.

GFAP is highly expressed in fibrous (white matter) astrocytes and increased in response to central nervous system (CNS) injury. Immunohistochemical staining for GFAP is beneficial for visualizing problem areas of injured brain tissue. Astrocytic expression and function is first detectable by 20 weeks gestation in human brain tissue, indicative of a supportive role for astrocytes in the perinatal brain during intra-uterine ischemic injury (Roessmann and Gambetti, 1986).

MBP is the essential protein required for formation of CNS myelin by mature oligodendrocytes. In the developing rat, this process does not begin until after the first

postnatal week and continues to mature throughout development. It has been previously demonstrated that measures of MBP immuno-staining provide a reliable indication of oligodendroglial injury in the developing rodent brain (oligodendrocyte progenitors appear early in fetal development) (Liu et al., 2002). Inspection of these stains at PD21 provides semi-long-term information about the white matter development in brains of rats exposed to intrauterine ischemia.

Frozen brain sections were post-fixed in formalin or methanol (for MBP) and then cleared and dehydrated in a graded series of ethanol washes. Sections were then washed with 1% hydrogen peroxide to quench endogenous peroxidases and subsequently blocked with normal horse serum mixed with Triton X-100 (0.1%). Sections were then allowed to incubate with the primary antibody overnight. After rinsing and 30 minutes of incubation with the secondary antibody, the sections were rinsed again and incubated with an avidin-biotin complex (ABC, Vector Laboratories Inc., Burlingame, CA., USA.). The immunoreactivity is visualized with diaminobenzedine tetrahydrochloride (DAB, Vector Laboratories Inc.).

GFAP Cell Counting

Images of GFAP stained coronal sections (1.6 mm anterior to bregma) were taken at 400X magnification in the areas of the corpus callosum and cingulum with a Spot Flex Camera (Diagnostic Instruments, Sterling Heights MI) attached to a Leica ATC 2000 microscope (Leica, Buffalo, NY) and Spot 4.5 software (Diagnostic Instruments, Sterling Heights MI). For the corpus callosum, images were taken on either side of the midline, alternating between left and right hemispheres. For the

cingulum, the tip of the cingulate peak was positioned to the lowest point of view in the microscope field so the image would reflect a portion of the cingular white matter projections just superior to the peak, again alternating hemispheres between sections.

Reactive astrocytes denoted by their enlarged cell bodies and highlighted processes were marked on a grid overlay of the collected images using Adobe Photoshop CS2 ver 9.0.2 computer software, which enables removal of the background image, making the marks within the grid easy to count.

White Matter Thickness, Ventricle Area and Densitometry

Whole brain images of MBP stained sections (1.6 mm anterior and 2.5 mm posterior to bregma) were taken with a Spot Flex Camera (Diagnostic Instruments, Sterling Heights MI) attached to a Leica GZ6E stereoscope (Leica Microsystems, Richmond Hill ON, Canada) and Spot 4.5 software (Diagnostic Instruments, Sterling Heights MI). All measurements were obtained using Image J ver 1.41 (reference - Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009.) calibrated with a Kodak No. 3 Calibrated Step Tablet scanned with an Epson Expression 1680 Professional scanner.

Both anterior and posterior (mid brain) sections were measured for corpus callosum thickness and cingulate peak thickness. Measurements for the corpus callosum were taken at the mid-line of each section and cingulum thickness was measured from the most visibly superior fibers in the peak to the most inferior of the corpus callosum in both hemispheres of each section and averaged for each animal (see Figure 2).

MBP stained sections (1.6 mm anterior to bregma) were also used to measure ventricular areas and densitometry of myelin staining. Left and right ventricular areas were determined and averaged for each animal. For densitometry, a 0.035 mm^2 area of the corpus callosum immediately beside the midline was measured. The same area of the dorsal cortex beside the midline that did not show MBP reactivity was also measured to determine background staining levels. This background level was then subtracted from the optical density of the corpus callosum, in the same section. This accounts for variability in staining results that can occur between different batches of immunohistochemistry.

Enzyme-Linked Immunosorbent Assays (ELISA)

Six frozen whole brain samples from each group were sent for quantification of GFAP and MBP by ELISA to the Laboratory of Marc Del Bigio, Department of Pathology, University of Manitoba. ELISA's were performed according to a procedure developed in the Del Bigio Lab as previously described (Khan et al., 2006).

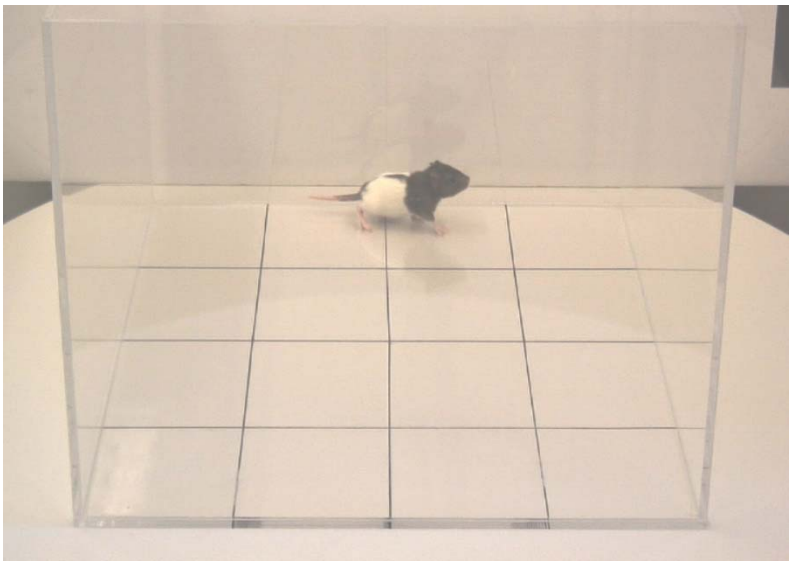
Statistical Analyses

Where possible, an experimenter blinded to the treatment groups scored the behavioural testing of the rat pups. Due to the observable appearance of growth restricted animals and the requirement for an experienced and consistent animal researcher to carry out the testing of neonatal rats, it was not always possible to be blind. The video-monitored tests of righting, acceleration righting and open field were scored by a blind experimenter.

Between group comparisons were analyzed with SPSS Statistics using the student t-test, one-way Analysis of Variance (ANOVA), two-way ANOVA and the Levine tests for equality of error variances or homogeneity of variances. Tukey HSD or Games-Howell post hoc tests were used where necessary, (SPSS Statistics 17.0; SPSS Inc., Chicago, IL, U.S.A.). Data are expressed as mean ± standard deviation (SD). A p value of ≤ 0.05 was considered to be statistically significant.

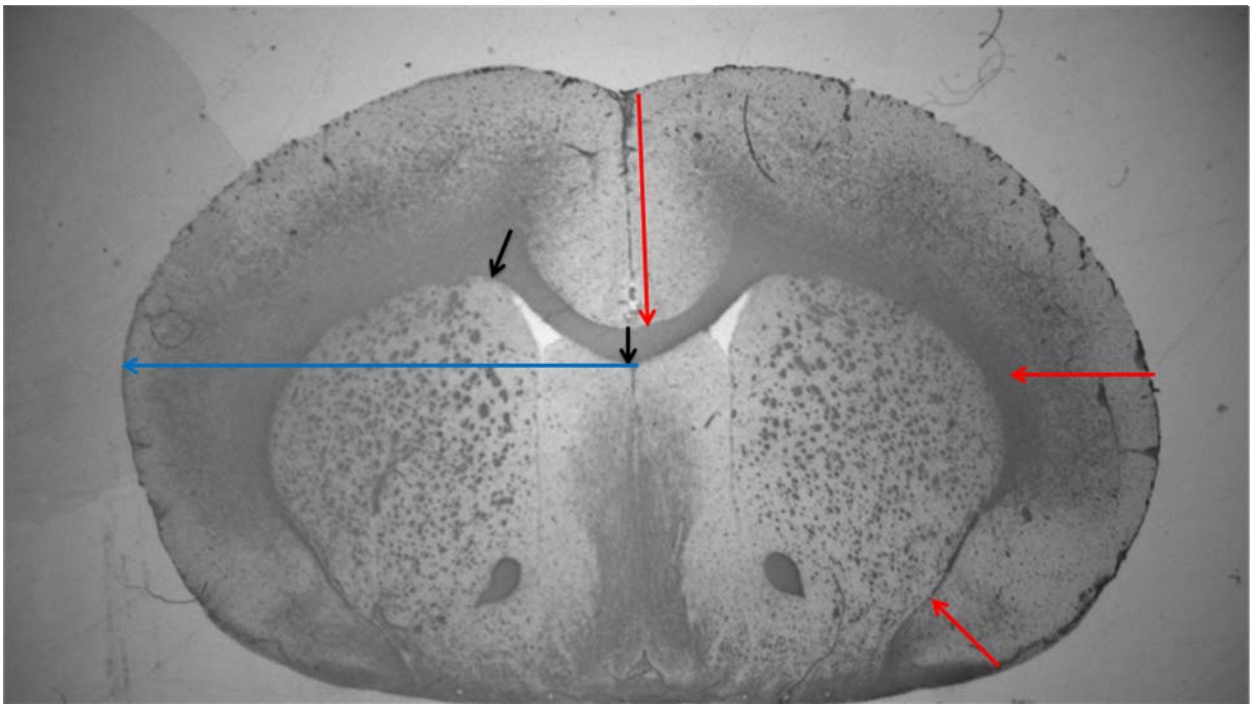
Methods Figures

Figure 1



Legend: Open Field Behavioral Test Apparatus

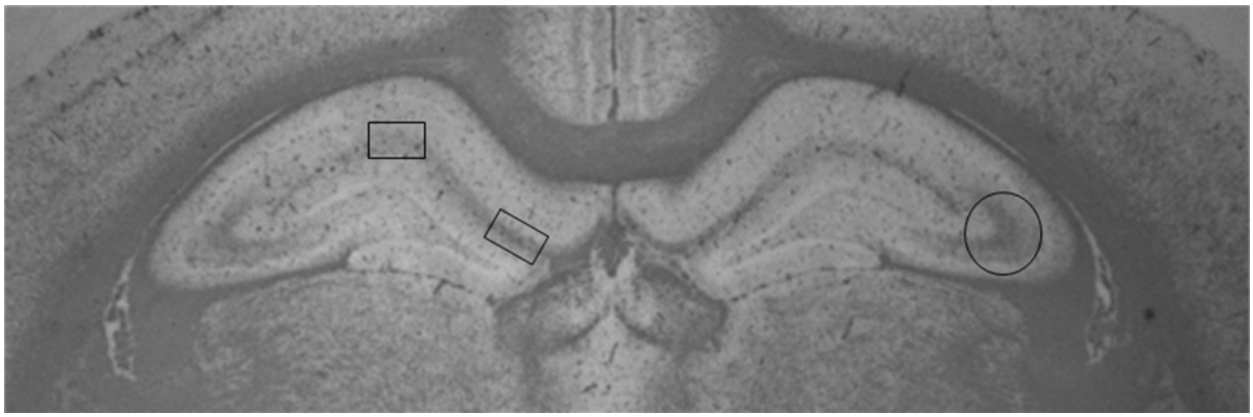
Figure 2



Legend: MBP stained anterior section depicting brain measurements

The black arrows illustrate where the measurements for the corpus callosum and cingulate peak were made. The red arrows indicate cortical thickness measurements and the blue is hemisphere width.

Figure 3



Legend: MBP stained posterior section depicting areas of hippocampus where cell counts were made. The two areas counted in the CA1 are shown in the boxes and the CA3 region is shown in the circle.

Results

Exclusions, Mortality and Growth Restriction

The BUAL surgery was tolerated well by all rats in the study. Sixteen litters were born on E23 and one on E22. The early litter was excluded on the basis of pup prematurity and ~24 hours less time spent in the ischemic environment. One dam chewed sutures through the muscular layer and was euthanized. The pups from her litter were fostered to other dams with same age litters and likewise for one other litter in the study, which only produced four pups.

Absolute mortality of the BUAL model cannot be ascertained without counting the pups in each horn during pregnancy and then performing cesarean sections, in lieu of natural delivery, which was not part of the protocol for this study. In order to estimate mortality, Graph Pad Prism and one-way ANOVA with Tukey post hoc were used to compare litter sizes (viable pups only) between all four experimental groups as well as a naïve group of litters born in our colony during the same time period. There was no difference in litter size between SHAM, SHAM+B and the naïve group ($p > 0.05$; mean ± SD - 13.0 ± 3.4; 13.0 ± 2.9; 14.4 ± 2.2) and no difference between BUAL and BUAL+B litter size ($p > 0.05$; mean ± SD - 6.7 ± 3.6; 6.6 ± 2.6). BUAL and BUAL+B litters were significantly smaller than all three control groups ($p < 0.01$; Figure 4). The differences found in litter size suggest a significantly higher mortality rate among BUAL fetuses with no effect due to gestational broccoli consumption. However, the SHAM and SHAM+B groups had small sample sizes of 6 and 5 respectively, compared to samples of 11, 10

and 9 in naïve, BUAL and BUAL+B groups. Because the SHAM/SHAM+B litters obtained provided enough pups for the study, no further SHAM surgeries were performed, but the small sample sizes for these groups would have limited statistical power in this case. A one-way ANOVA comparison of SHAM, SHAM+B and naïve newborn weights was also done to assess any effect the SHAM surgery or broccoli supplementation may have had on growth and development of SHAM fetuses. No significant differences were observed in this comparison (Naïve: 6.37 ± 0.35 g; SHAM: 6.41 ± 0.29 g; SHAM+B: 6.18 ± 0.21 g; n = 20, 16 and 16 respectively; $p > 0.05$)

During the course of the study, some dams designated for BUAL/BUAL+B groups gave birth to litters too small in number for the long-term developmental based comparison of this study. Small litter size could introduce confounds like less competition at feeding and more maternal attention for the pups of these litters. As an alternative to exclusion, in the interest of animal conservation, those litters with less than 5 pups that could not be fostered to alternate dams, were used for other research projects, and are included here only as part of the mortality estimate.

Of the 10 BUAL/BUAL+B litters used in this study (54 pups), 2 animals were excluded for being overweight at birth by our criteria (i.e. not IUGR by definition, see Methods). For the results and discussion sections of this thesis, BUAL groups are referred to as IUGR because only growth restricted pups were included in these comparisons.

The calculation of Cephalization Index (CI), determined as a ratio of head circumference to weight on PD1 (individual litters) revealed a ‘brain sparing’ effect of our model. For these litters BUAL resulted in a mean birth weight of 4.41 ± 0.20 g,

significantly less than the SHAM newborns used in this comparison, whose birth weight was 6.71 ± 0.12 g ($p < 0.0001$). In addition, the BUAL group had significantly smaller head circumference (3.42 ± 0.07cm) compared to the SHAM animals (4.17 ± 0.06 cm; $p < 0.0001$). Values of CI were thus, SHAM = 0.62 ± 0.01 cm/g and BUAL = 0.79 ± 0.02 cm/g; $p < 0.0001$). In this regard, growth restriction due to BUAL in the rat is asymmetric and comparable to IUGR as described clinically.

Weight at birth, PD7, PD14 & PD21

Daily weight gain was recorded to ensure pups were receiving adequate nutrition. A cessation in weight gain and failure to thrive was observed in one pup after PD14 and the animal was euthanized (early data [up to PD14] from this pup is included in this study, but late behaviour and weight data points are missing). Two-way ANOVA of birth weight indicated a significant main effect of treatment (BUAL surgery or SHAM), ($p < 0.0001$), no effect of diet (broccoli sprout supplementation or chow), ($p = 0.203$) and no interaction ($p = 0.536$). For pup weight at PD7, 14 and 21, similar results were obtained, a main effect of treatment ($p < 0.0001$ for all time points), an effect of diet on PD7 ($p = 0.018$), but not on PD14 or PD21 ($p = 0.231$; 0.626 respectively) and no interaction ($p = 0.737$ (PD7); 0.861 (PD14); 0.373 (PD21)). The Levine test was significant at all ages ($p \leq 0.029$). One-way ANOVA with the Games-Howell post hoc test supported the above results on birth weight and weight gain for the rat pups. Growth restricted pups (IUGR) weighed significantly less than SHAM pups throughout the study with no difference in these results when the dams were fed broccoli sprouts (Table 1 and Figure 5).

Further investigation of group growth patterns revealed IUGR and IUGR+B pups had a significantly higher fold-increase in weight by PD21 than SHAM or SHAM+B pups. Two-way ANOVA resulted in a significant effect of treatment ($p < 0.0001$), no effect of diet ($p = 0.053$) and no interaction ($p = 0.219$) (Figure 6).

Neurobehavioural/Reflex and Maturation Assessment

For all testing: n = 16, SHAM; 20, SHAM+B; 27, IUGR; 25, IUGR+B. Two-way ANOVAs showed a main effect of treatment in every test used to evaluate the pups (Table 2). A main effect of treatment for the righting reflex occurred for every day of testing (PD3-7) (Figure 7). A main effect of diet was observed in forelimb grasping, hind limb placing and cliff aversion and an interaction was observed in the forelimb grasping and posture tests (Table 1). Similar to the weight data, with the exception of gait, all tests were significant for the Levine statistic (overall, $p \leq 0.043$; data not shown). Therefore means were compared using one-way ANOVA and the Games-Howell post hoc test where an effect of diet or an interaction was observed.

Forelimb Grasp reflex: There was a significant difference in performance between IUGR and all other groups ($p < 0.0001$), no difference between SHAM and SHAM+B groups ($p = 0.914$), and no difference between IUGR+B and both SHAM groups ($p \geq 0.174$) (Figure 8a).

Hind limb Placing Response: For this test, IUGR animals were delayed compared to SHAM groups ($p < 0.0001$) and IUGR+B also showed poorer performance than SHAM groups ($p \leq 0.029$). Although animal behaviour was not returned to the levels of SHAM in this test, IUGR+B animals were still significantly improved over IUGR ($p = 0.009$)

(Figure 8b).

Cliff Aversion: Surprisingly, cliff aversion revealed a difference in behaviour between SHAM groups ($p < 0.0001$). IUGR animals were delayed compared to both SHAM groups ($p \leq 0.017$), and IUGR+B animals were improved over IUGR ($p < 0.0001$). However, IUGR+B were also significantly different from the SHAM group ($p < 0.0001$) and no different from SHAM+B ($p = 0.174$) (Figure 8c).

Posture: Attainment of a mature posture was delayed for IUGR animals compared to SHAM and SHAM+B ($p < 0.0001$). Broccoli did not have a significant effect on SHAM ($p = 0.972$), but did improve attainment of normal posture for the IUGR+B animals over IUGR ($p = 0.037$). IUGR+B were still delayed compared to SHAM groups ($p < 0.0001$) (Figure 8d).

Reflex and maturation testing indicated that growth restricted pups were significantly delayed in attaining the reflexes as well as showing signs of maturation compared to sham controls. Broccoli supplementation improved performance/outcome in some of these tests.

Open Field Behaviour

Two-way ANOVA of open field data showed a main effect of treatment in ambulation, head lifts and defecation, a main effect of diet in head lifts only and an interaction in ambulation, head lifts and grooming (Table 2). A comparison of means by one-way ANOVA and Tukey HSD or Games-Howell post hoc tests showed IUGR animals were significantly more active than SHAM groups in ambulation ($p \leq 0.002$) with no difference in activity between the SHAM and SHAM+B groups ($p = 0.105$). Broccoli-

treated IUGR animals performed significantly different from IUGR animals with respect to ambulation ($p = 0.001$) and were not different from SHAM ($p \geq 0.115$) (Figure 9a). Similarly for head-lifting behaviour, IUGR animals were overactive in comparison to SHAM groups ($p < 0.0001$). Broccoli-treated animals were less active than IUGR ($p = 0.027$) and performed no differently than SHAM ($p = 0.056$) (Figure 9b).

Two-way ANOVA shows a significant interaction for grooming. One-way comparison showed no differences between any of the groups for grooming ($p \geq 0.194$). Likewise, defecation showed a main effect of treatment, but no differences with one-way comparisons ($p \geq 0.064$). This discrepancy is probably due to the very low occurrence of grooming or defecation within any of the groups.

Open field testing on PD21 indicated an increased activity level in IUGR animals with respect to parameters of ambulation and head lifts compared to sham controls. Supplementation with broccoli sprouts significantly improved these field behaviours. Open field results further suggest, by two-way interactions that the effects of broccoli on some behaviours may depend on treatment conditions.

Histological Evaluation

It must be noted that some of the pups that accomplished the behavioural portion of this study are not included in the histological portion due to a complication of fixation, which resulted in a complete loss of tissue from those litters. Also, some brain sections successfully obtained were not suitable for measurements and cell counting due to problems with staining and/or sectioning (some sections were too anterior or posterior from our intended locations to make valid comparisons). Excluded sections varied

somewhat between methods of analysis, but despite these difficulties in histology, sample sizes of at least 8 brain sections per experimental group for all measures in the study were included.

Brain Size Measurements

IUGR and SHAM groups were used for basic measurements of brain size to ensure there were no differences due to growth restriction in general and were analyzed by the student t-test. Hemispheric measurements of width revealed no differences between SHAM and IUGR brain size in either the anterior or posterior planes evaluated (anterior: SHAM 6.43 ± 0.18 mm, n = 10; IUGR 6.53 ± 0.21 mm, n = 15; $p > 0.05$; posterior: SHAM 7.0 ± 0.14 mm, n = 10; IUGR 6.87 ± 0.26 mm, n = 13; $p > 0.05$). Cortical thickness measurements were the same in all three planes at the dorsal and lateral positions. However, with respect to inferior cortical development, the anterior plane showed IUGR cortical thickness to be significantly greater than SHAM and the posterior plane showed IUGR cortical thickness to be significantly less than SHAM. The middle plane showed no differences (Table 4). Finally, there was no difference in the length of the hippocampus between SHAM and IUGR groups (SHAM = 3.49 ± 0.13 mm, n = 9; IUGR = 3.46 ± 0.06 mm, n = 12; $p > 0.05$). The differences in inferior cortical development are interesting. It is possible the posterior development of the IUGR inferior cortex was inhibited by the injury and perhaps compensated for in the anterior inferior cortical development. However, further research in this area must be done to draw conclusions. Measurements of brain growth, such as thickness and volumes of specific structures over time would provide more reliable explanations.

Hippocampal Cell Counting

CA1 cell counts were summated and reported as an overall average for each section. Two-way ANOVA for CA1 cell counts resulted in a main effect of treatment ($p < 0.0001$), a main effect of diet ($p < 0.0001$) and a significant interaction ($p < 0.002$). A comparison of means by one-way ANOVA and Tukey's post hoc test showed no difference between SHAM and SHAM+B groups (99.4 ± 5.6, $n = 8$; 100.8 ± 5.9, $n = 8$; $p = 0.973$). IUGR+B had significantly more cells in CA1 than IUGR (85.2 ± 8.2, $n = 13$; 70.3 ± 5.4, $n = 13$; $p < 0.0001$), however IUGR+B counts did not reach the level of SHAM animals ($p < 0.0001$) (Figure 10a). CA3 cell counts resulted in a main effect of treatment ($p < 0.0001$), no effect of diet ($p = 0.169$), but a significant interaction ($p < 0.03$). Again, one-way ANOVA comparisons show no difference between SHAM groups (152.8 ± 19.5, 148.7 ± 13.3; $p = 0.946$). IUGR+B had significantly more cells than IUGR (134.7 ± 11.8, 117.2 ± 17; $p = 0.029$ and were not statistically different from SHAM ($p = 0.057$) (Figure 10b).

The IUGR group had significantly less neurons in the CA1 and CA3 areas of the hippocampus than SHAM control groups. Broccoli sprout supplementation significantly improved cell counts in both areas. Figure 11 shows photomicrographs of the CA1 area in all four experimental groups.

GFAP Cell Counting

GFAP cell counts are reported as the mean number of cells counted per area per group. For the corpus callosum, two-way ANOVA resulted in a main effect of treatment, a main effect of diet and a significant interaction ($p < 0.0001$, $p < 0.0001$ and $p = 0.001$,

respectively, Figure 12a). Mean cell counts were 11.8 ± 3.0, 10.1 ± 2.1, 18.9 ± 1.8 and 11.9 ± 2.4 for SHAM, SHAM+B, IUGR and IUGR+B respectively.

For the cingulum area, two-way ANOVA gave a main effect of treatment and of diet ($p < 0.0001$, $p = 0.002$), but no interaction ($p = 0.088$) (Figure 12b). Mean counts in the same order as above were 29.4 ± 6.0, 26.8 ± 5.6, 42.1 ± 4.9 and 33.8 ± 4.5. Although the interaction was not significant by two-way, one-way ANOVA shows the IUGR+B group is significantly different from IUGR ($p = 0.005$).

IUGR animals had more reactive astrocytes in both the corpus callosum and cingulum white matter areas than SHAM controls. Broccoli sprout treated IUGR animals had significantly less reactive astrocytes in these areas than IUGR alone (See Figure 13 photomicrograph of astrocytic reactivity).

White Matter Thickness, Densitometry and Ventricle Areas

Corpus callosum (cc) thickness measurements in the anterior rat brain revealed a main effect of treatment ($p < 0.0001$), a main effect of diet ($p = 0.009$) and no interaction ($p = 0.318$) by two-way ANOVA. Comparison of means by one-way ANOVA and Tukey's post hoc test showed IUGR animals had a significant reduction in cc compared to SHAM ($p = 0.001$). The IUGR+B group showed significant improvement in cc thickness compared to IUGR ($p = 0.033$) and was not different from SHAM in this measure (0.494) (Figure 14a). The posterior brain cc thickness measurements gave a main effect of treatment ($p < 0.0001$), but no effect of diet ($p = 0.279$) and no interaction ($p = 0.14$). One-way comparisons show no difference between IUGR+B and control groups ($p \geq 0.096$), but also no difference compared to IUGR ($p = 0.205$) (Figure 14b).

Thickness measurements of the cingulate peak (cp) in the anterior rat brain resulted in a main effect of treatment ($p < 0.0001$), a main effect of diet ($p < 0.0001$) and a significant interaction ($p = 0.001$). Mean comparisons by one-way ANOVA and Tukey's post hoc revealed the cp thickness of the IUGR group is reduced significantly compared to SHAM controls ($p < 0.0001$). The IUGR+B cp measure was significantly improved over IUGR ($p < 0.0001$) and not different from SHAM controls ($p = 0.983$) (Figure 14c). Posterior cp thickness gave a main effect of treatment ($p < 0.0001$), a main effect of diet ($p < 0.0001$), but no interaction ($p = 0.115$). As with the anterior sections, one-way mean comparisons showed a significant reduction in posterior cp thickness of the IUGR group compared to controls ($p < 0.002$) and again, IUGR+B was significantly improved over IUGR ($p < 0.0001$) and not different from SHAM controls ($p = 0.999$) (Figure 14d).

Densitometry measurements gave a main effect of treatment ($p = 0.002$), no effect of diet ($p = 0.087$) and a significant interaction ($p = 0.025$). A one-way ANOVA with Tukey post hoc showed a significantly weaker MBP signal in IUGR sections compared with SHAM ($p = 0.001$). IUGR+B had a significantly stronger MBP signal compared with IUGR ($p = 0.016$) and was not different compared to SHAM controls (0.721) (Figure 15).

Area measurements of ventricles gave a main effect of treatment ($p < 0.0001$), no effect of diet ($p < 0.071$), but a significant interaction ($p < 0.004$) and significant Levene test ($p = 0.003$). One-way ANOVA with the Games-Howell post hoc test revealed significantly larger ventricle areas in IUGR sections compared to SHAM ($p < 0.0001$). The broccoli supplemented IUGR group had significantly smaller ventricle

areas than the IUGR group ($p = 0.025$) although not to the level of control (Figure 16).

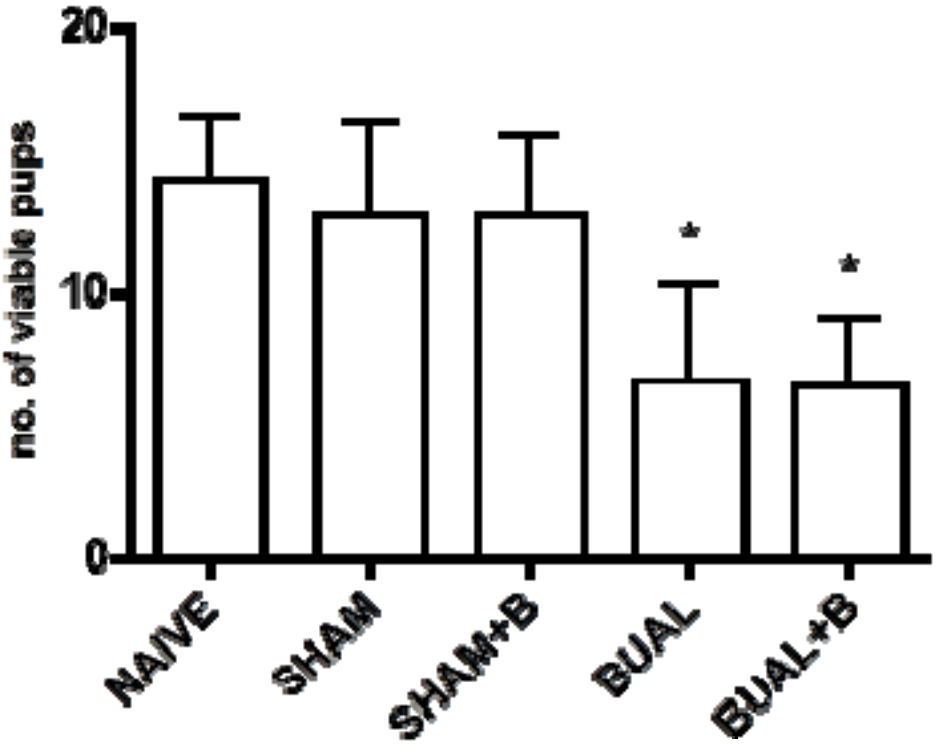
Histological examination with MBP in both the anterior and posterior areas of the rat brain show IUGR animals to be deficient in axon abundance and myelin development compared to SHAM controls. Overall, broccoli supplementation improved the abundance of axons and production of myelin in growth restricted animals. No significant difference in SHAM due to broccoli was observed in any of these measures. Figures 17, 18 and 19 show photomicrographs of MBP stained sections illustrating differences in the parameters measured and reported here.

ELISAs

Two-way ANOVA of analysis by ELISA of GFAP showed no significant differences in protein concentration between groups (mean ± SD: 0.17 ± 0.09; 0.13 ± 0.06; 0.16 ± 0.09 and 0.19 ± 0.09 mg GFAP/g for SHAM, SHAM+B, IUGR and IUGR+B respectively). The MBP analysis showed a main effect of treatment ($p < 0.0001$), but no other significant effects (2.76 ± 0.3; 2.64 ± 0.2; 2.17 ± 0.3 and 1.93 ± 0.53 mg MBP/g; see Figures 20a & b).

Results – Figures/Tables/Legends

Figure 4



Legend: The graph shows a comparison of litter size as an indication of BUAL mortality. The number of viable pups per litter were counted in the four experimental groups as well as a naïve group of litters from our breeding colony (n = 11, 6, 5, 10 and 9 litters, respectively).

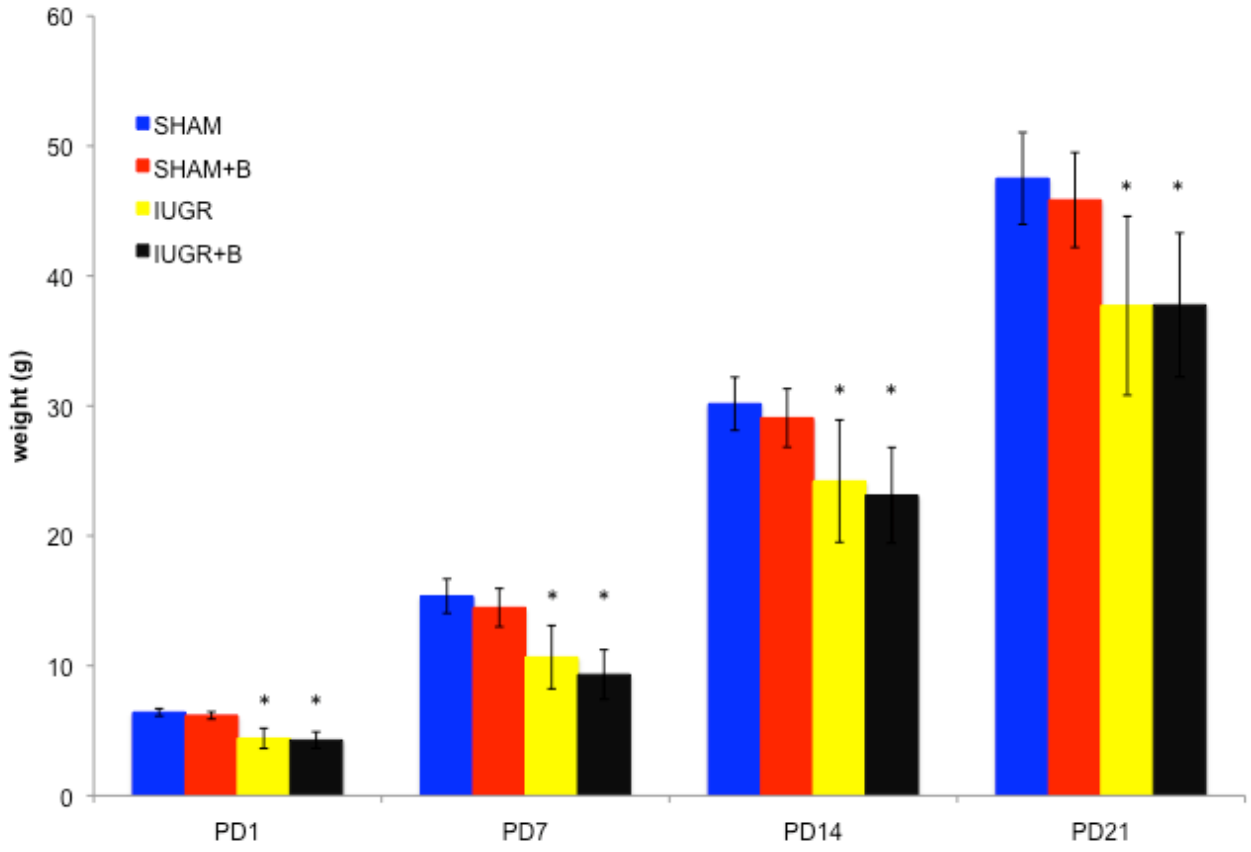
* Both BUAL and BUAL+B litters were significantly smaller in the number of living pups than all other groups ($p < 0.01$ by one-way ANOVA; data is expressed as mean ± SD).

Table 1

	SHAM	SHAM+B	IUGR	IUGR+B
n	16	20	27	25
PD1	6.41 ± 0.29	6.17 ± 0.28	*4.41 ± 0.78	*4.27 ± 0.64
PD7	15.36 ± 1.32	14.47 ± 1.48	*10.66 ± 2.43	*9.33 ± 1.90
PD14	30.16 ± 2.01	29.07 ± 2.25	*24.21 ± 4.71	*23.12 ± 3.68
PD21	47.51 ± 3.54	45.85 ± 3.65	*37.71 ± 6.89	*37.77 ± 5.54

Legend: Means ± SD of group weights on postnatal days 1, 7, 14 and 21 are shown in Table 1, corresponding with the bar graph in Figure 2.

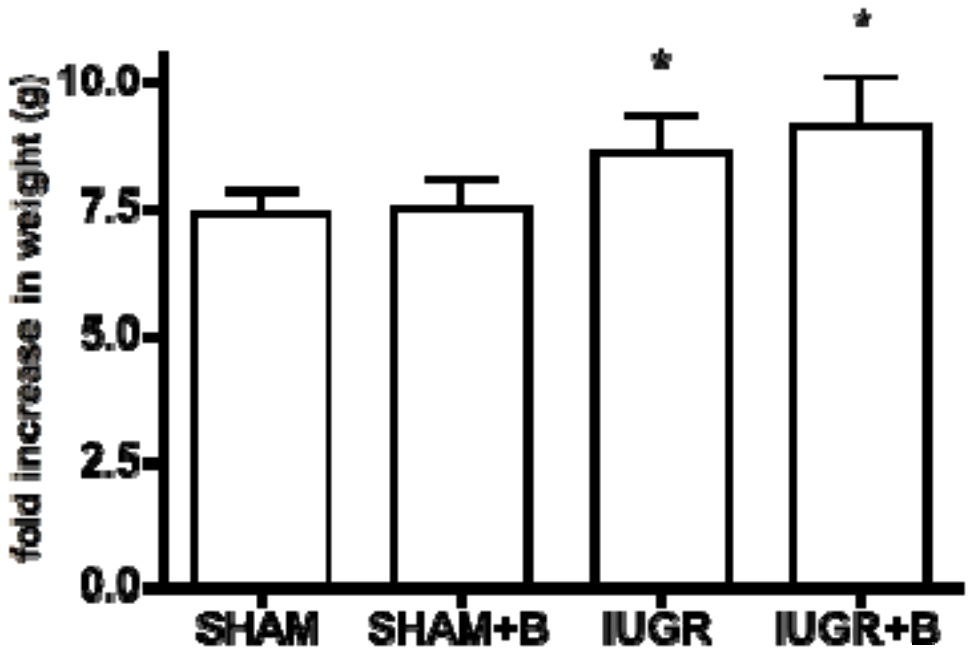
Figure 5



Legend: The graph illustrates group weights at birth (PD1) and postnatal days 7, 14 and 21 (pathology day) for all four experimental groups. Broccoli supplementation had no effect on birth weight or weight gain in either supplemented group.

* IUGR and IUGR+B groups weighed significantly less than SHAM groups on each day that was analyzed (main effect of treatment by two-way ANOVA, $p < 0.0001$ at all time points; data is expressed as mean ± SD as shown in Table 1).

Figure 6



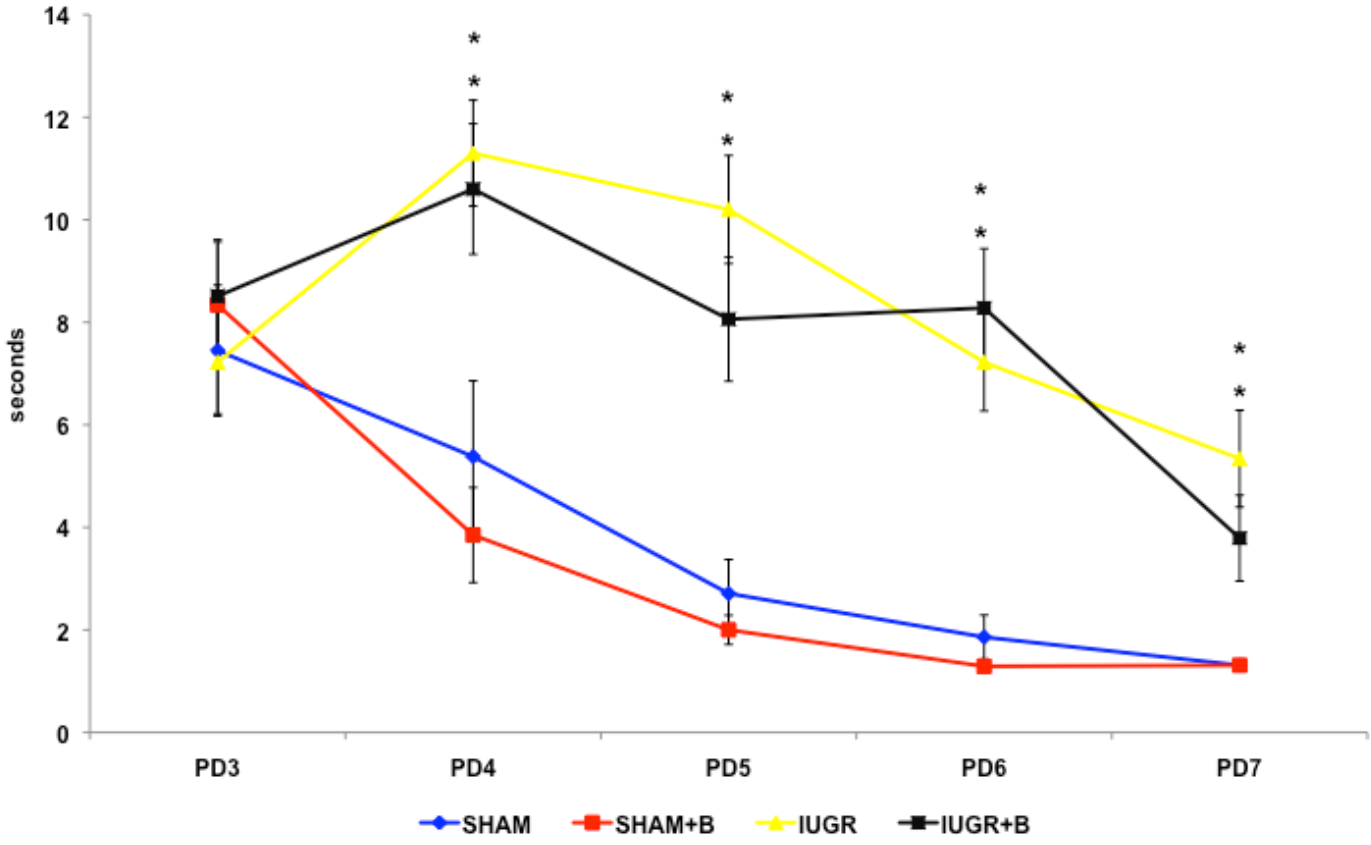
Legend: The graph shows a measure of growth by group fold-increase in weight, calculated by: weight PD21 / weight PD1. Broccoli did not have an effect on growth. * IUGR and IUGR+B groups fold-increase in weight was significantly greater than SHAM groups (main effect of treatment by two-way ANOVA, $p < 0.0001$; n = 16, 20, 27, 24; data is expressed as mean ± SD).

Table 2

Test	Group Means				Main Effects (p-value)		
	SHAM	SHAM+B	IUGR	IUGR+B	Treatment	Diet	Interaction
n	16	20	27	25			
Righting PD7 (s)	1.3 ± 0.4	1.3 ± 0.3	5.2 ± 4.8	4.6 ± 4.8	0.000	0.715	0.708
Forelimb grasp (PD)	2.9 ± 0.3	3.0 ± 0.3	4.6 ± 1.0	3.3 ± 0.9	0.000	0.000	0.000
Hind limb grasp	4.3 ± 0.9	3.8 ± 0.8	6.8 ± 2.2	6.1 ± 2.1	0.000	0.124	0.848
Hind limb placing	5.5 ± 1.2	5.1 ± 0.9	8.1 ± 1.4	6.8 ± 1.5	0.000	0.002	0.118
Cliff Aversion	6.6 ± 0.7	5.0 ± 1.0	7.7 ± 1.5	5.5 ± 0.9	0.002	0.000	0.291
Gait	7.8 ± 1.1	7.1 ± 1.0	8.9 ± 1.3	8.8 ± 1.1	0.000	0.121	0.229
Posture	14.3 ± 0.6	14.4 ± 0.6	16.5 ± 1.3	15.7 ± 0.9	0.000	0.067	0.024
Eye Opening	16.2 ± 0.5	16.4 ± 0.6	16.6 ± 0.9	16.7 ± 0.6	0.028	0.360	0.903
Auditory	11.9 ± 0.7	12.4 ± 0.8	13.7 ± 1.2	14.0 ± 0.7	0.000	0.094	0.547
Acceleration-righting	15.3 ± 1.0	15.6 ± 0.9	18.0 ± 2.1	17.2 ± 1.0	0.000	0.413	0.085

Legend: Table 2 shows means ± SD as well as p-values by two-way ANOVA for early reflex, behaviour and maturation tests. Two-way ANOVA of the data clearly show a main effect of treatment in all tests. There is an effect of diet in forelimb grasp, hind limb placing and cliff aversion and an interaction in forelimb grasp and posture tests. All tests with the exception of gait resulted in a significant Levine test ($p \leq 0.043$; data not shown).

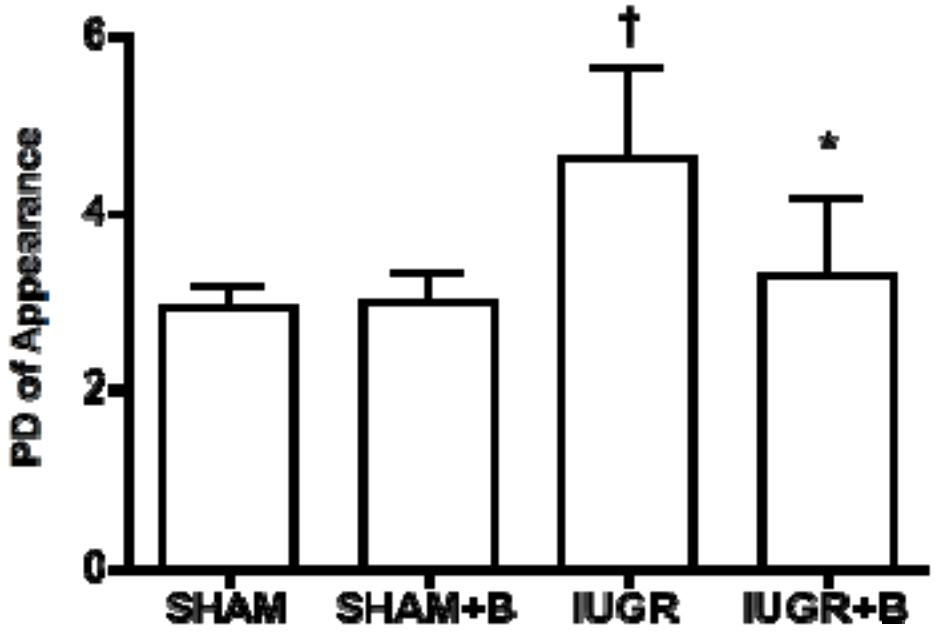
Figure 7



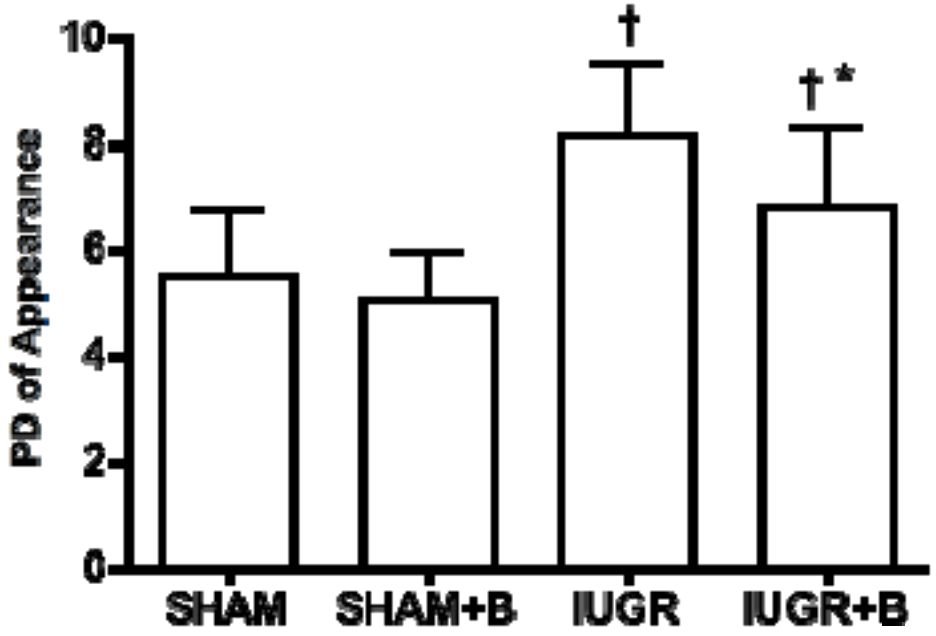
Legend: The graph shows the result of the righting reflex in seconds by postnatal day. A consistent main effect of treatment was observed for righting from PD3 – PD7, i.e. both IUGR and IUGR+B groups were significantly slower in accomplishing this task than SHAM groups. * two-way ANOVA on each day; $p < 0.0001$; $n = 16, 20, 27, 25$; data is expressed as mean ± SD).

Figure 8

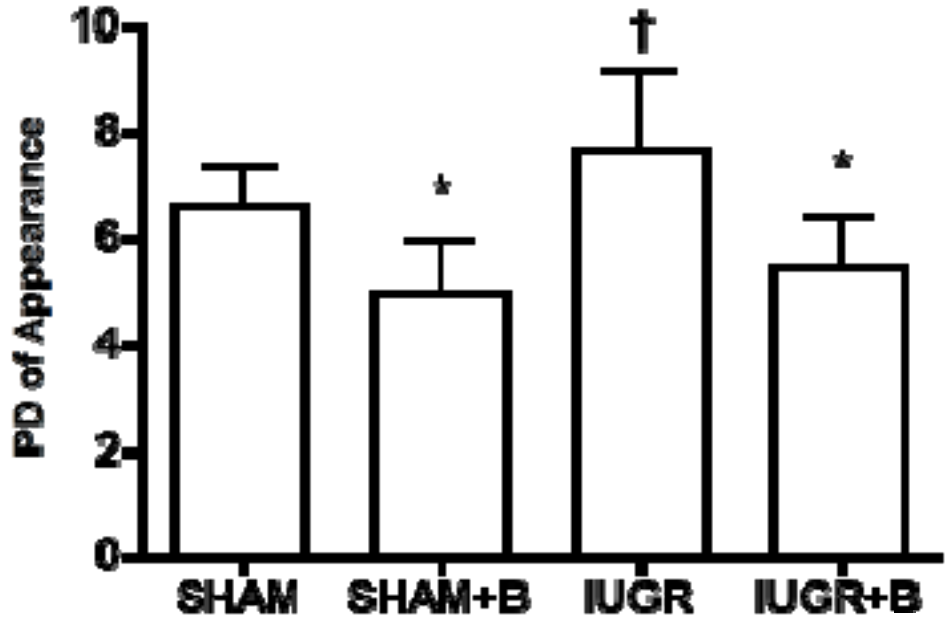
a) Forelimb grasping reflex



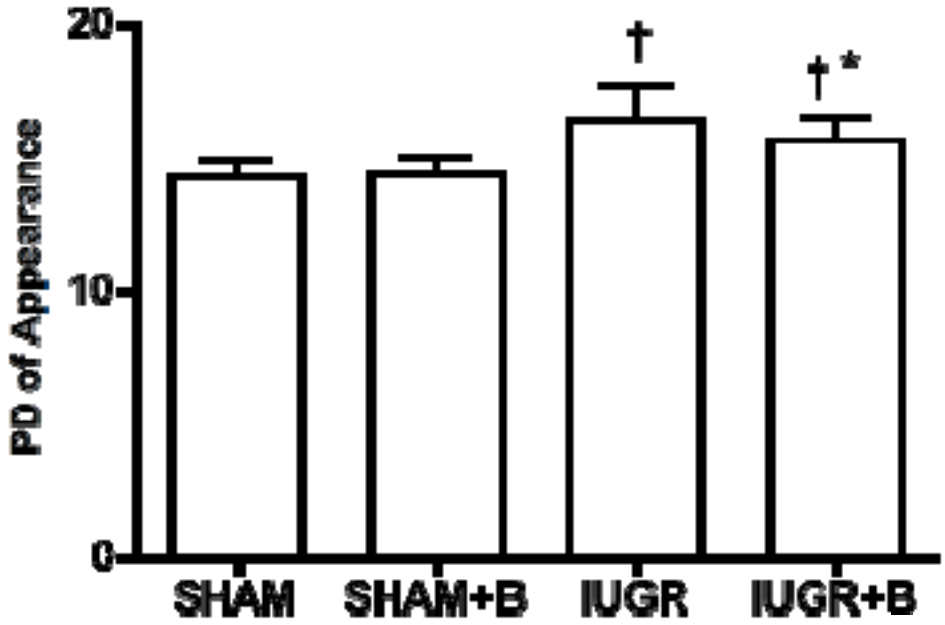
b) Hind limb placing reflex



c) Cliff aversion reflex



d) Attainment of normal posture



Legend: Figure 5 shows examples of reflex test results from our neurologic/maturation test battery. The IUGR group was delayed compared to SHAM in attaining all reflexes examined (Table 2). Tests of a) forelimb grasping, b) hind limb placing, c) cliff aversion and d) posture, indicate broccoli treatment may obviate some deficits caused by placental insufficiency.

By one-way ANOVA: forelimb grasp - † significantly different from SHAM $p < 0.0001$, * significantly different from IUGR $p < 0.0001$; hind limb placing - † $p \leq 0.029$, * $p = 0.009$; cliff aversion - † $p = 0.017$, * significantly different from SHAM and IUGR $p < 0.0001$, posture - † $p < 0.0001$, * $p = 0.037$; data is expressed as mean ± SD.

(See Table 2 for two-way ANOVA p-values)

Table 3

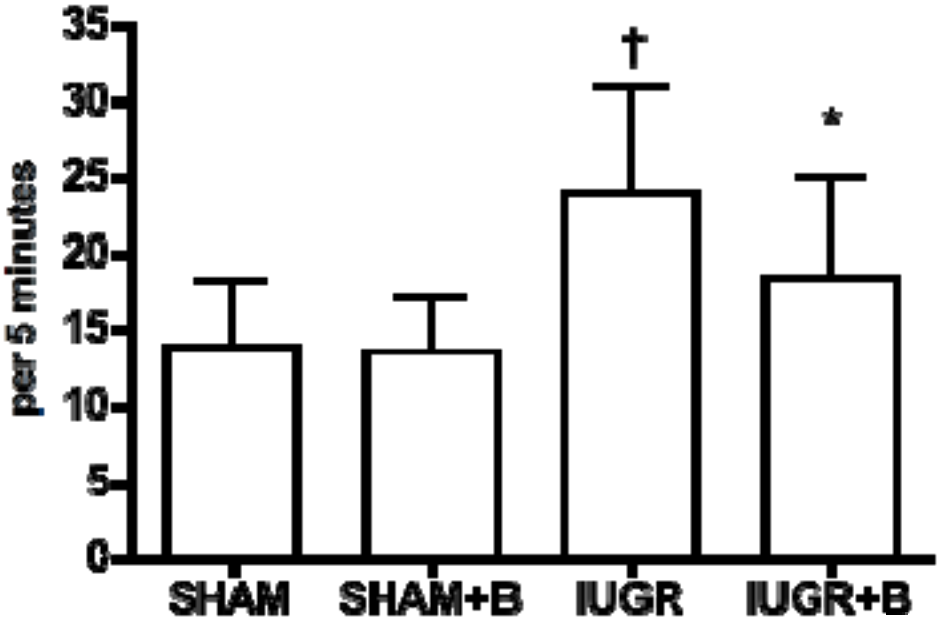
Test	Group Means				Main Effects (p-value)		
	SHAM	SHAM+B	IUGR	IUGR+B	Treatment	Diet	Interaction
n	**16**	**20**	**26**	**24**			
Ambulation (squares)	67.6 ± 23.8	87.1 ± 21.6	115 ± 24.1	86.0 ± 29.5	0.000	0.390	0.000
Head lifts	13.9 ± 4.4	13.5 ± 3.8	24.0 ± 6.8	18.5 ± 7.2	0.000	0.022	0.045
Rearing	35.3 ± 9.6	38.4 ± 8.8	36.8 ± 9.6	33.0 ± 12.2	0.393	0.850	0.127
Grooming	1.7 ± 1.4	0.9 ± 0.8	1.0 ± 0.8	1.3 ± 0.8	0.519	0.182	0.010
Defecation	2.3 ± 1.0	1.7 ± 1.1	1.3 ± 1.4	1.2 ± 1.6	0.011	0.268	0.447

Legend: The Table shows results from the open field behavioural test, data are reported as mean ± SD. Two-way ANOVA shows a main effect of treatment for ambulation, head lifts and defecation. A main effect of diet is observed in head lifts and an interaction in ambulation, head lifts and grooming. The Levene statistic was significant for head lifts and grooming ($p \leq 0.02$; data not shown).

Figure 9

a) Ambulation

b) Head-lifting



Legend: Figures 6a and b show open field measures of activity. IUGR animals showed hyperactivity in both ambulation and head lifting behaviour. Broccoli treated IUGR animals behaved no differently from SHAM controls in either measure.
By one-way ANOVA: ambulation - † significantly different from SHAM $p < 0.0001$, * significantly different from IUGR $p = 0.001$; head lifting - † $p < 0.0001$, * $p = 0.027$; data is expressed as mean ± SD.
(See Table 3 for two-way ANOVA p-values)

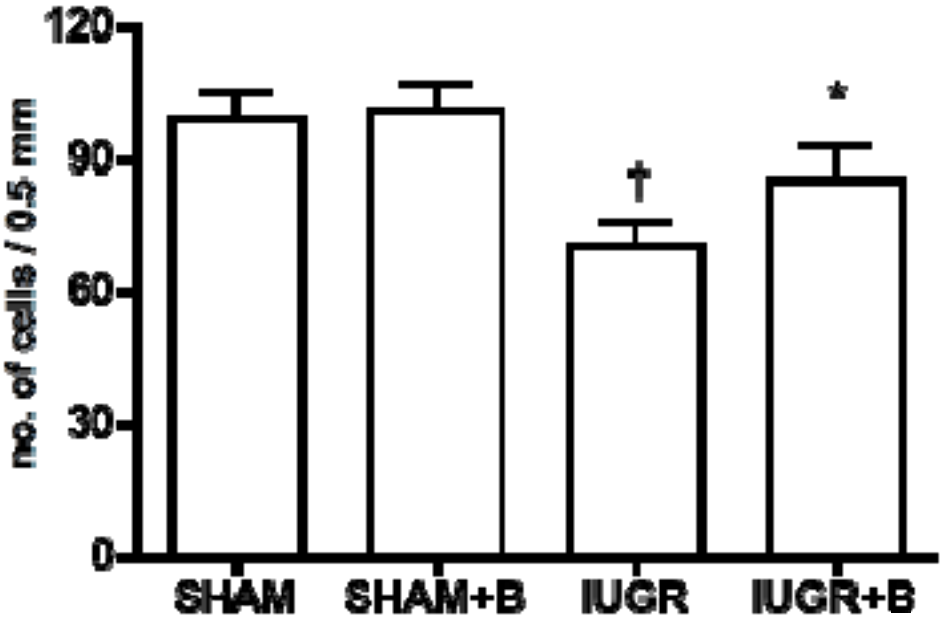
Table 4

PLANE	CORTEX	SHAM			IUGR			
		mean	SEM	n	mean	SEM	n	p-value
anterior	dorsal	2.649	0.07	10	2.74	0.08	14	>0.05(ns)
	lateral	1.873	0.02	10	1.837	0.02	14	ns
	inferior	1.155	0.04	10	1.398	0.04	15	< 0.05
middle	dorsal	2.473	0.07	10	2.328	0.07	12	ns
	lateral	1.858	0.02	10	1.898	0.03	12	ns
	inferior	1.302	0.04	10	1.339	0.05	12	ns
posterior	dorsal	2.184	0.05	10	2.120	0.06	13	ns
	lateral	2.242	0.09	10	1.983	0.04	13	ns
	inferior	2.664	0.08	10	1.786	0.08	13	< 0.05

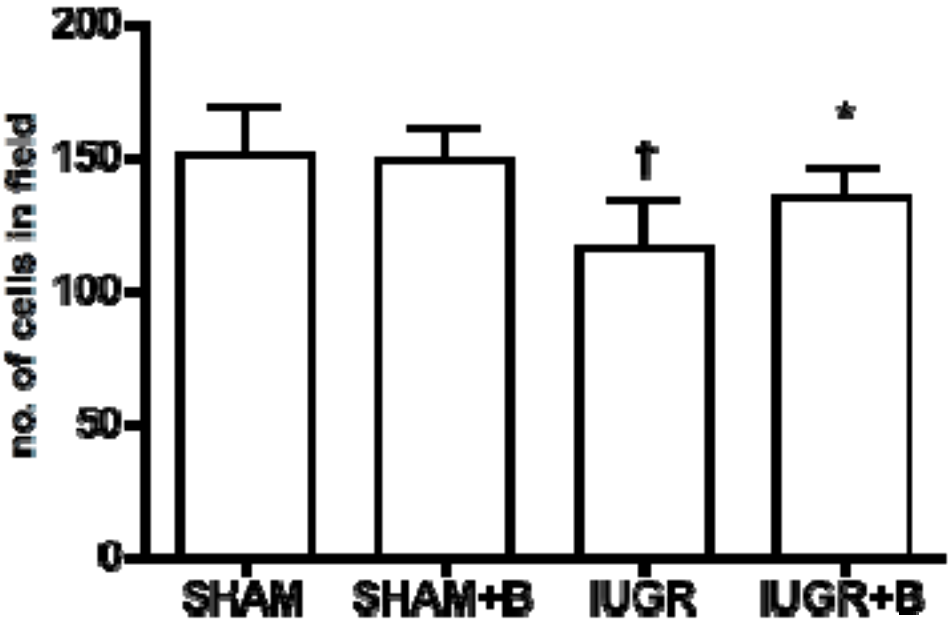
Legend: The Table shows means and SEM from the student's t-test of cortical thickness at three different areas across the anterior, middle and posterior planes of section. Inferior cortical development shows differences in the anterior and posterior planes only. In the anterior plane, inferior cortical development is thicker in the IUGR sections while in the posterior plane, inferior cortical development is thicker in the SHAM sections.

Figure 10

a) CA1

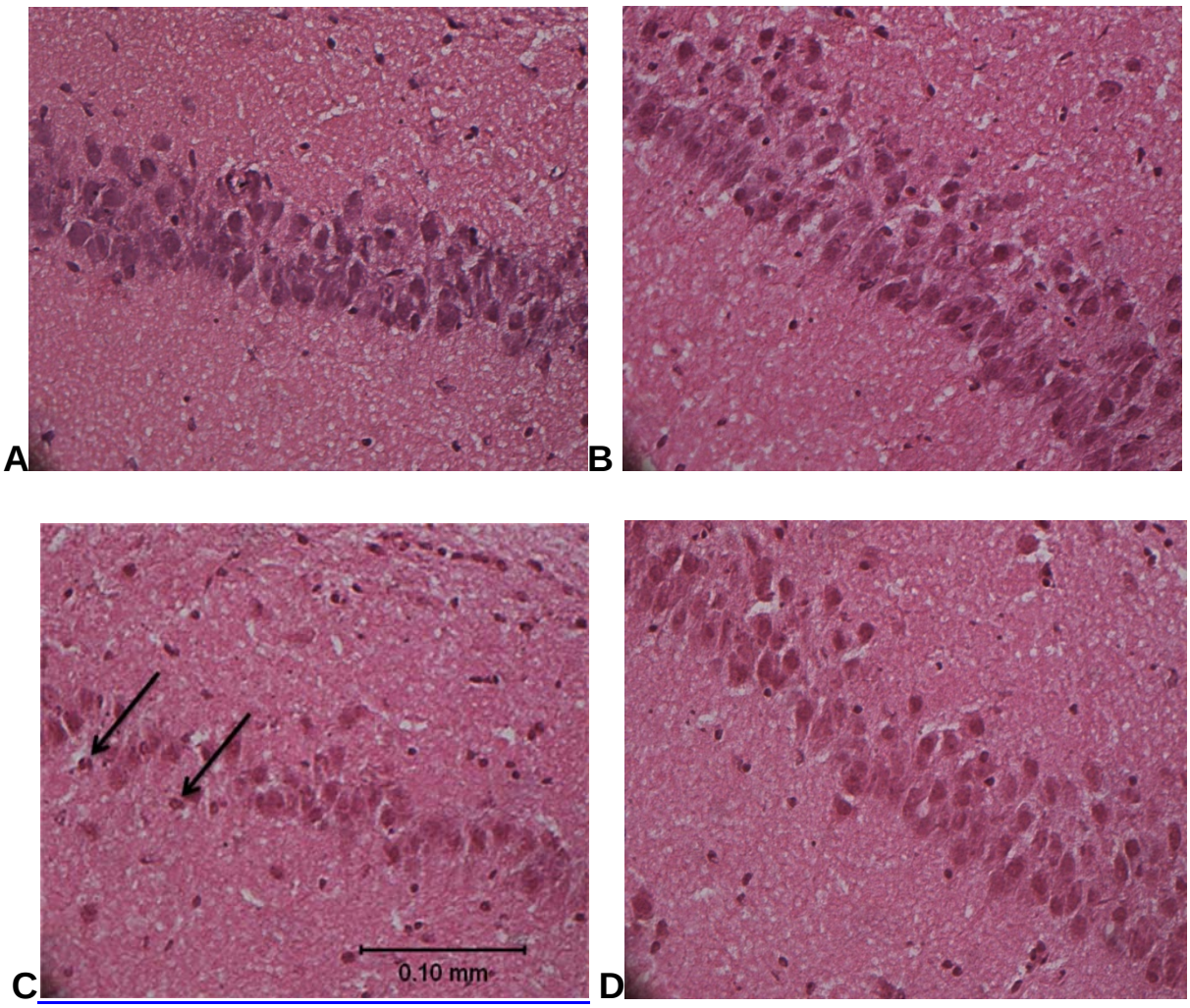


b) CA3



Legend: Figures 7a and b show IUGR cell counts in CA1 and CA3 of the hippocampus were significantly less than SHAM controls. Broccoli treatment increased cell counts significantly in IUGR animals - † different from SHAM $p < 0.0001$, * different from IUGR, CA1 $p < 0.0001$; CA3 $p = 0.029$ (CA1 counts are expressed per 0.5 mm and CA3 counts are per microscope field of view at 400X; n = 8, 8, 13 and 13 for SHAM, SHAM+B, IUGR and IUGR+B respectively, data is expressed as mean ± SD).

Figure 11

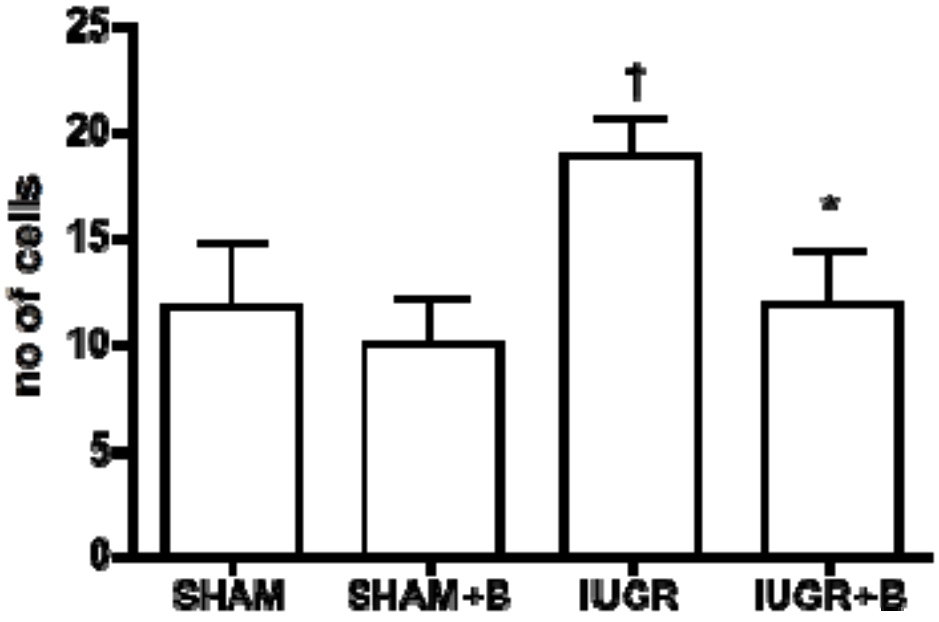


Legend: CA1 SHAM (A); SHAM+B (B); IUGR (C); IUGR+B (D)

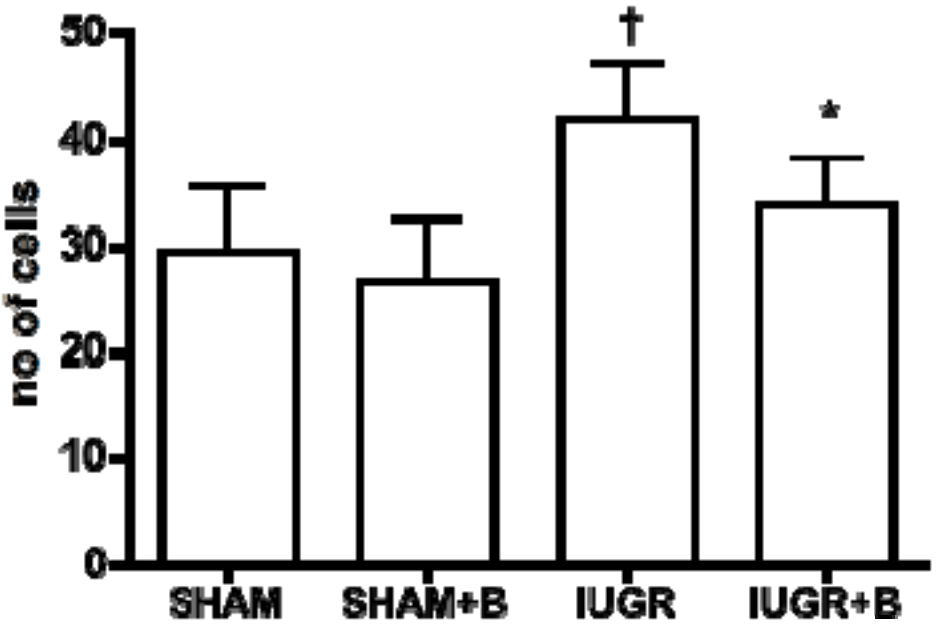
The images illustrate the observable reduction in pyramidal cells in the CA1 area of the IUGR section (slide C) compared with the other groups at PD21. Pyknotic cells are still visible in IUGR sections, indicated by the black arrows.

Figure 12

a) corpus callosum



b) cingulum

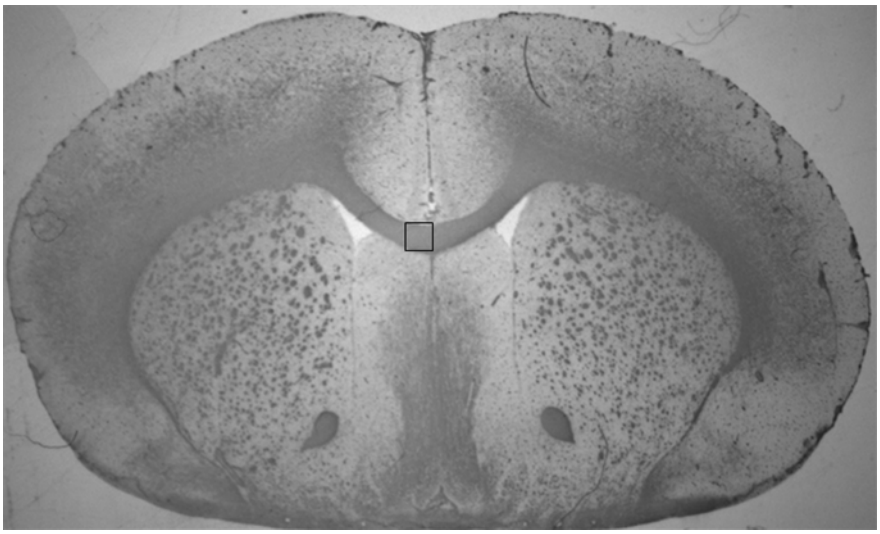


Legend: Figure 9a and b show cell counts for reactive astrocytes in the corpus callosum and cingular projections at PD21. Cells were counted from pictures taken at 400X magnification. IUGR animals had significantly more reactive astrocytes in both areas (main effect of treatment by 2-way ANOVA, $p < 0.0001$) compared with SHAM. Broccoli sprout supplemented groups had significantly less reactive astrocytes in both areas.

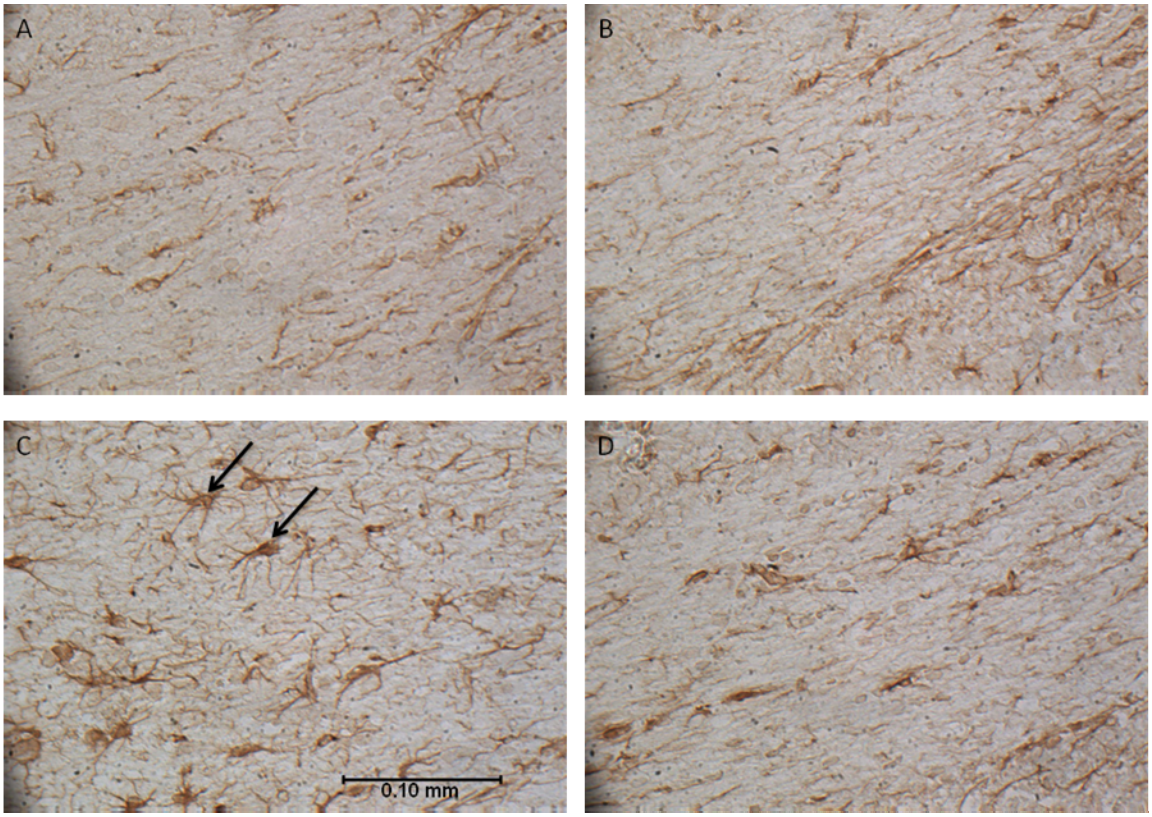
By one-way ANOVA - † different from SHAM, corpus callosum $p < 0.0001$; cingulum $p < 0.0001$; * different from IUGR, corpus callosum $p < 0.0001$; cingulum $p = 0.005$ ($n = 9, 12, 9$ and 12; data is expressed as mean ± SD).

Figure 13

a)



b)

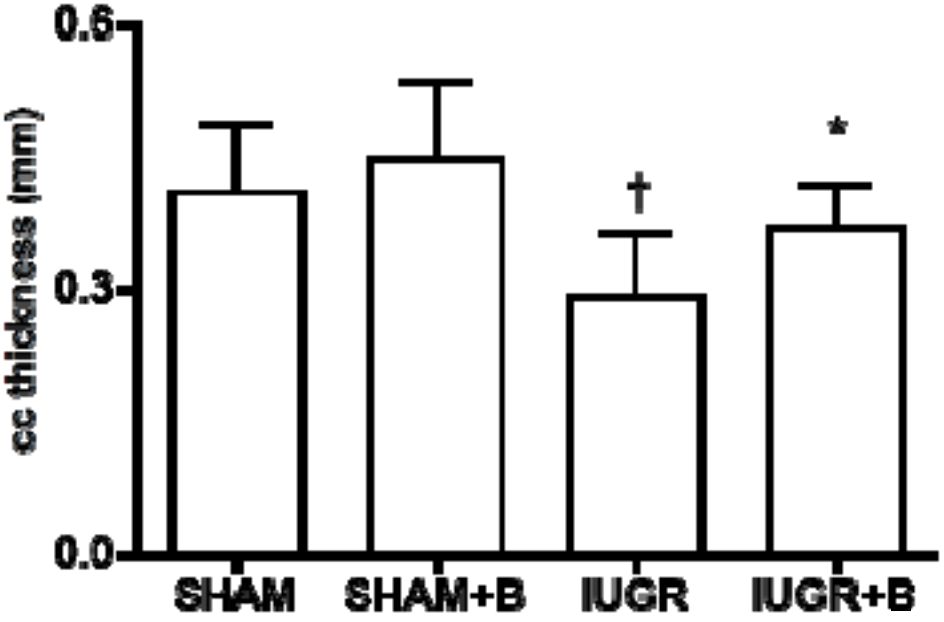


Legend: The anterior whole brain image **(a)** is stained with MBP for clarity, but indicates the area (black box) where GFAP images were taken for counting reactive astrocytes in the corpus callosum. The subsequent four images **(b)** depict the following groups: SHAM (A); SHAM+B (B); IUGR (C); IUGR+B (D)

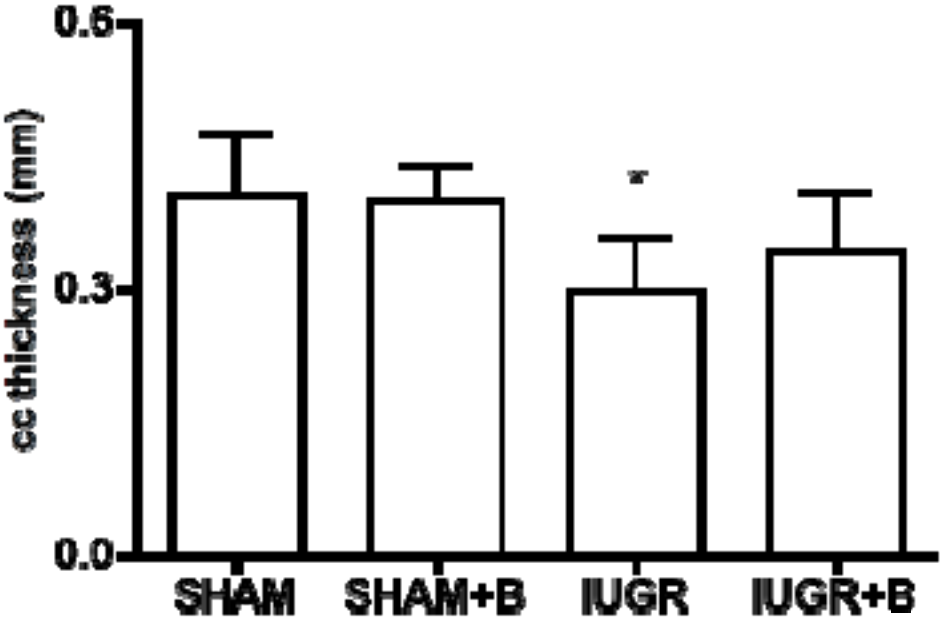
The photomicrographs (400X magnification) show more enlarged cell bodies and highlighted processes of reactive astrocytes (black arrows) in the corpus callosum of the IUGR image compared with the other three groups.

Figure 14

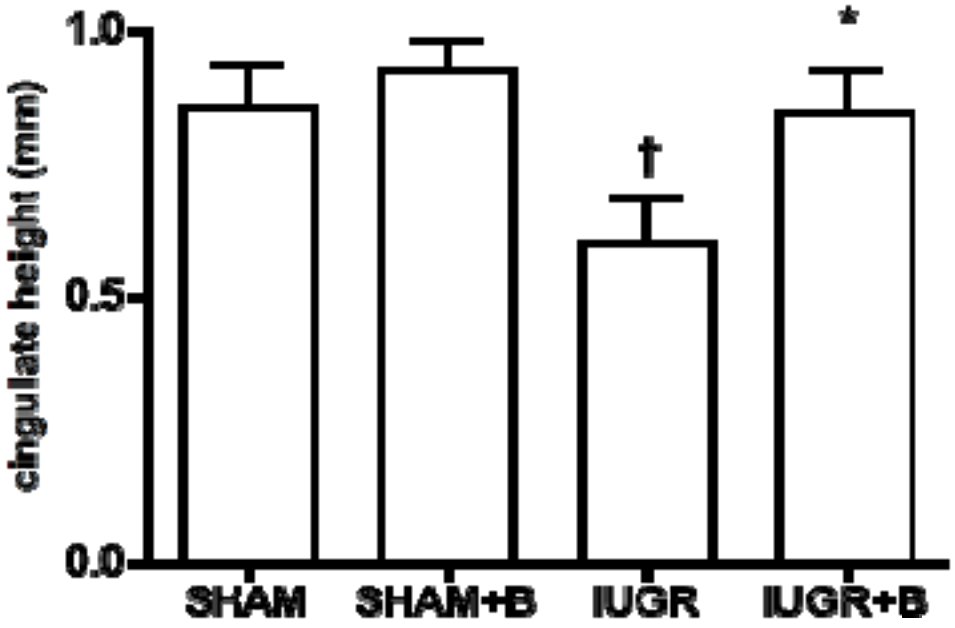
a) anterior corpus callosum



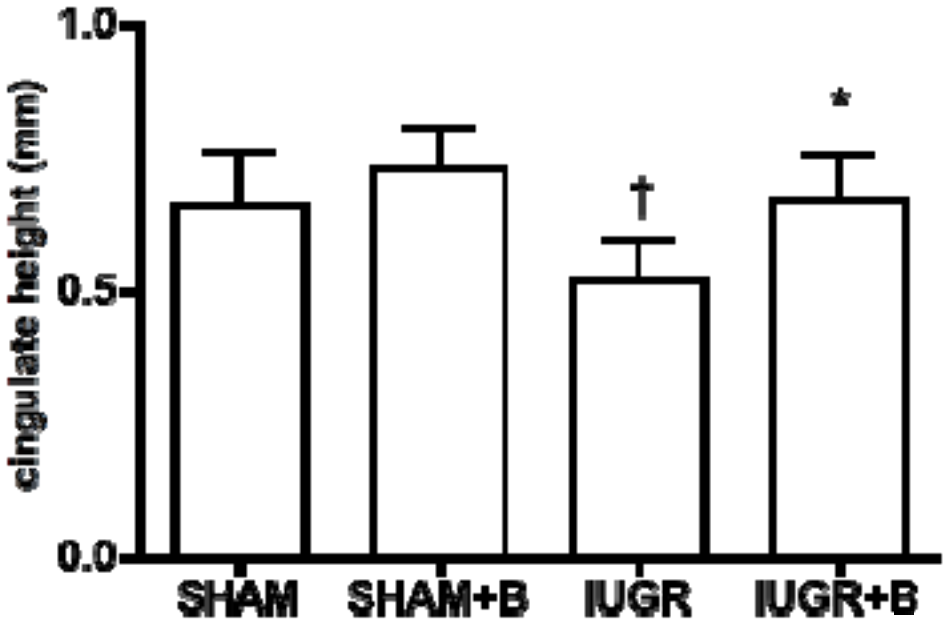
b) posterior corpus callosum



c) anterior cingulate peak



d) posterior cingulate peak



Legend: Graphs 11a and b show white matter measurements of the corpus callosum (cc) thickness of anterior and posterior sections of the brain at PD21. The cc shows thinning in both anterior and posterior areas of IUGR animals (main effect of treatment by two-way ANOVA $p < 0.0001$). Significant improvement due to broccoli was observed only in the anterior section (main effect of diet $p = 0.009$ by two-way ANOVA).

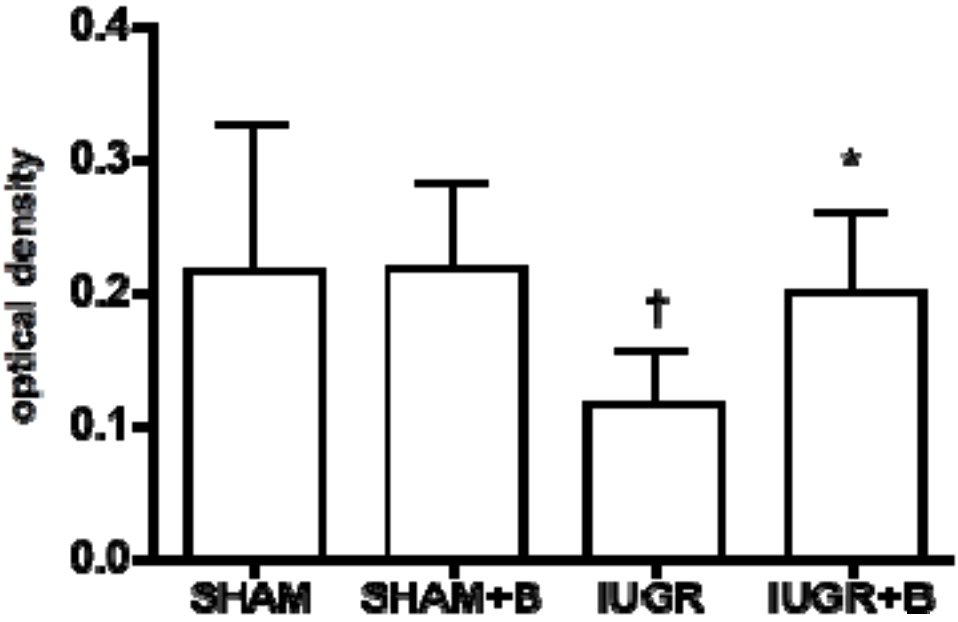
By one-way ANOVA: anterior cc - † different from SHAM $p = 0.001$, * different from IUGR $p = 0.033$; posterior cc - † $p = 0.001$ ($n = 9, 10, 14, 13$).

Graphs 9c and d show the height of the peak of the cingulum projections in both anterior and posterior sections at PD21. The cingulate peak (cp) was significantly smaller in both anterior and posterior brain areas of IUGR animals (main effect of treatment by two-way ANOVA $p < 0.0001$). Similarly, in both anterior and posterior areas, the cp of IUGR+B were significantly larger than IUGR (main effect of diet $p < 0.0001$).

By one-way ANOVA: anterior cp - † different from SHAM $p < 0.0001$, * different from IUGR $p < 0.0001$ ($n = 13, 12, 10$ and 10).

All data is expressed as mean ± SD.

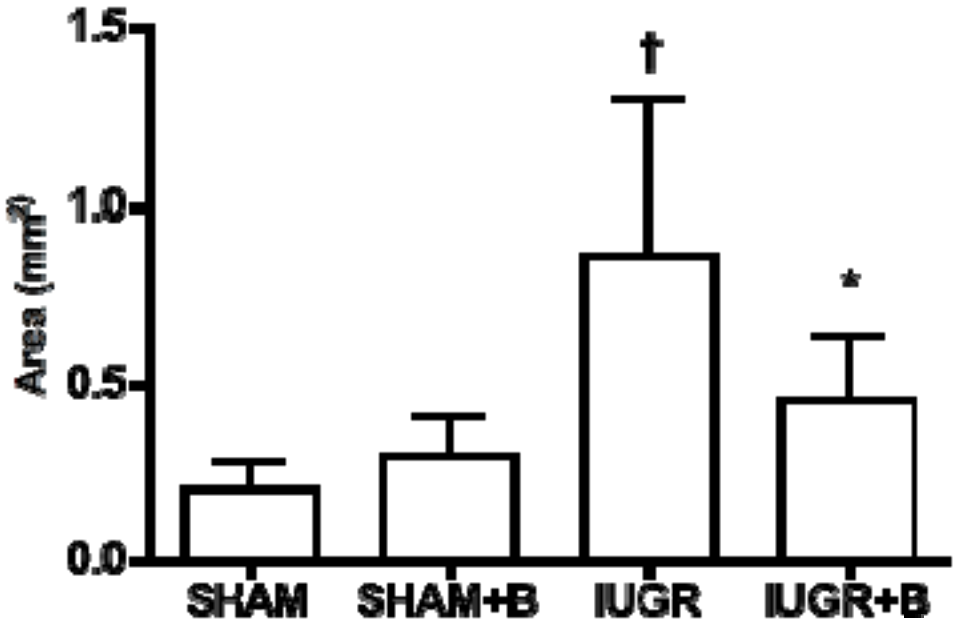
Figure 15



Legend: The graph shows mean optical density for MBP stained sections (1.6 mm anterior to bregma). MBP signal was significantly reduced in IUGR sections compared to SHAM (main effect of treatment by two-way ANOVA p = 0.002). IUGR+B section showed significant improvement in MBP optical density (significant interaction p = 0.025).

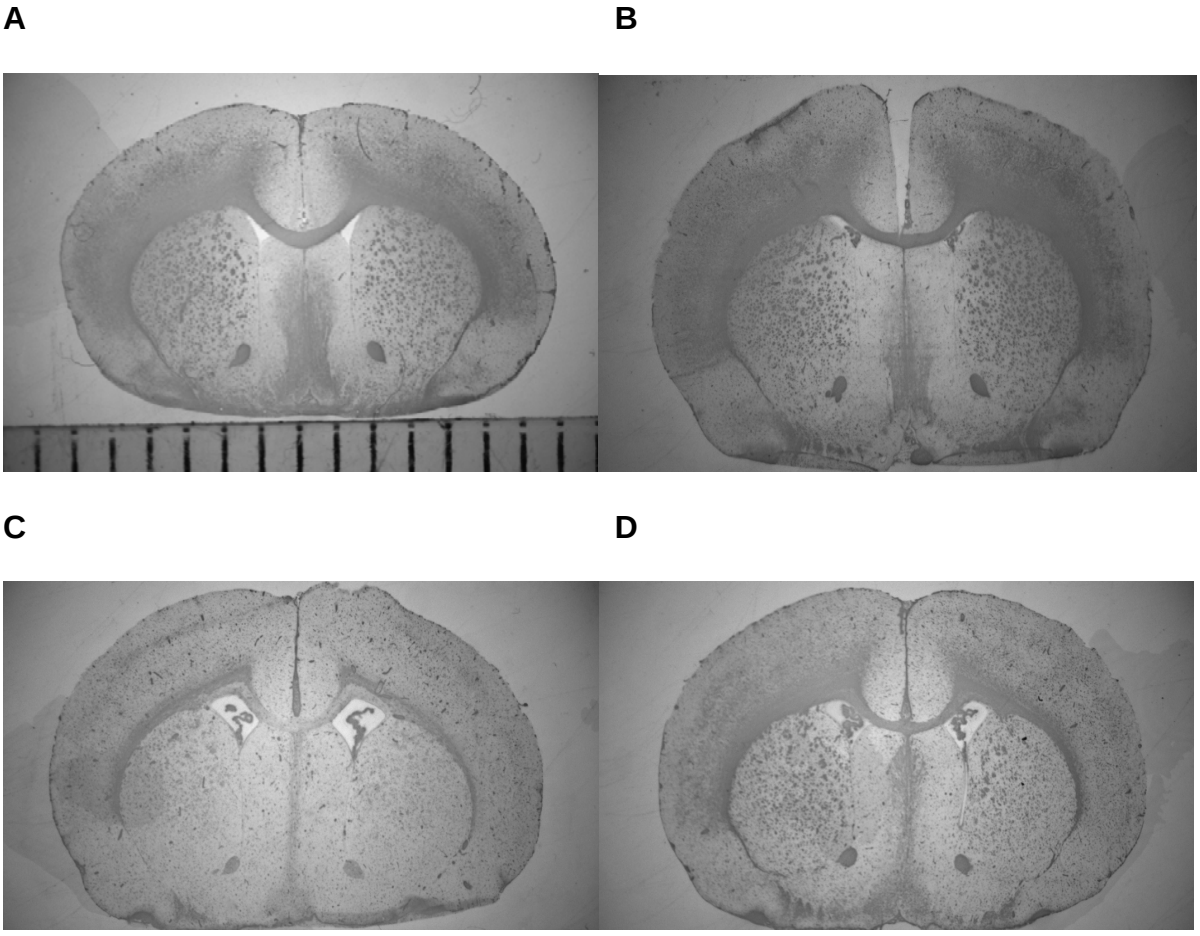
By one-way ANOVA - † different from SHAM p = 0.001, * different from IUGR p = 0.016 (n = 10, 9, 14 and 12; data is expressed as mean ± SD).

Figure 16



Legend: The graph shows significant ventricular dilation of IUGR animals compared to SHAM (main effect of treatment by two-way ANOVA $p < 0.0001$), measured by taking the area of the ventricle in anterior sections (1.6 mm anterior to bregma). Broccoli treated IUGR animals showed a 50% reduction in ventricle area compared to IUGR (significant interaction $p = 0.004$, the Levene statistic was also significant $p = 0.003$). By one-way ANOVA - † different from SHAM $p < 0.0001$, * different from IUGR $p = 0.025$ ($n = 11, 13, 14$ and 14; data is expressed as mean ± SD).

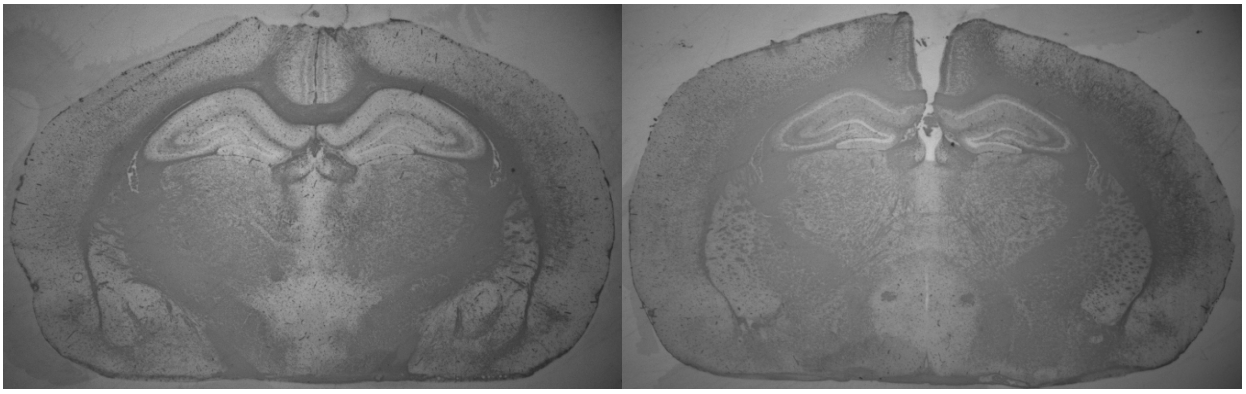
Figure 17



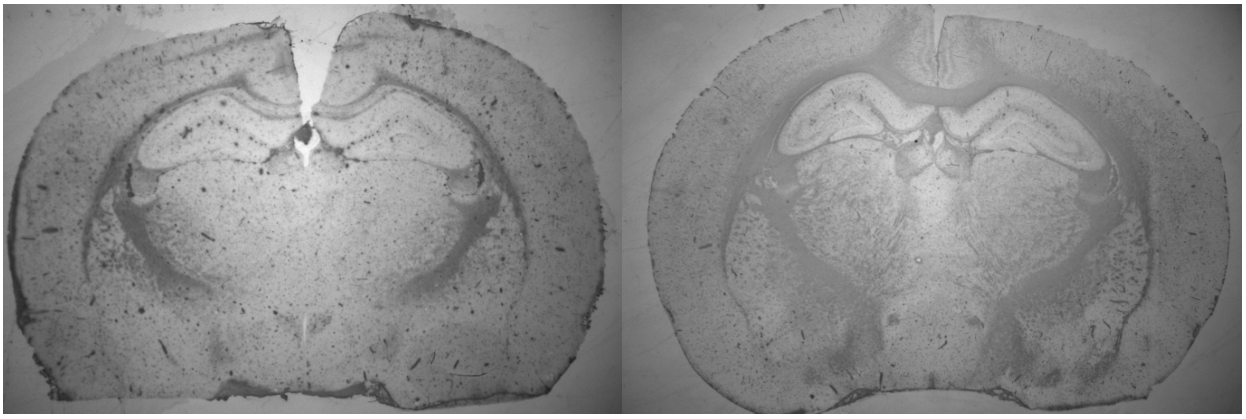
Legend: Photomicrographs show myelin basic protein (MBP) stained anterior brain sections, **A** SHAM, **B** SHAM+B, **C** IUGR, **D** IUGR+B (scale: each tick = 1 mm). The IUGR section shows the widespread weakness of the MBP signal and ventricular dilation in the IUGR animals compared to the other groups. Increased density of MBP staining can be seen in SHAM and SHAM+B images as well as the normal ventricle size for the rats at this stage (PD21). The IUGR+B image shows an improvement in myelination and ventricle size in comparison to IUGR.

Figure 18

A **B**

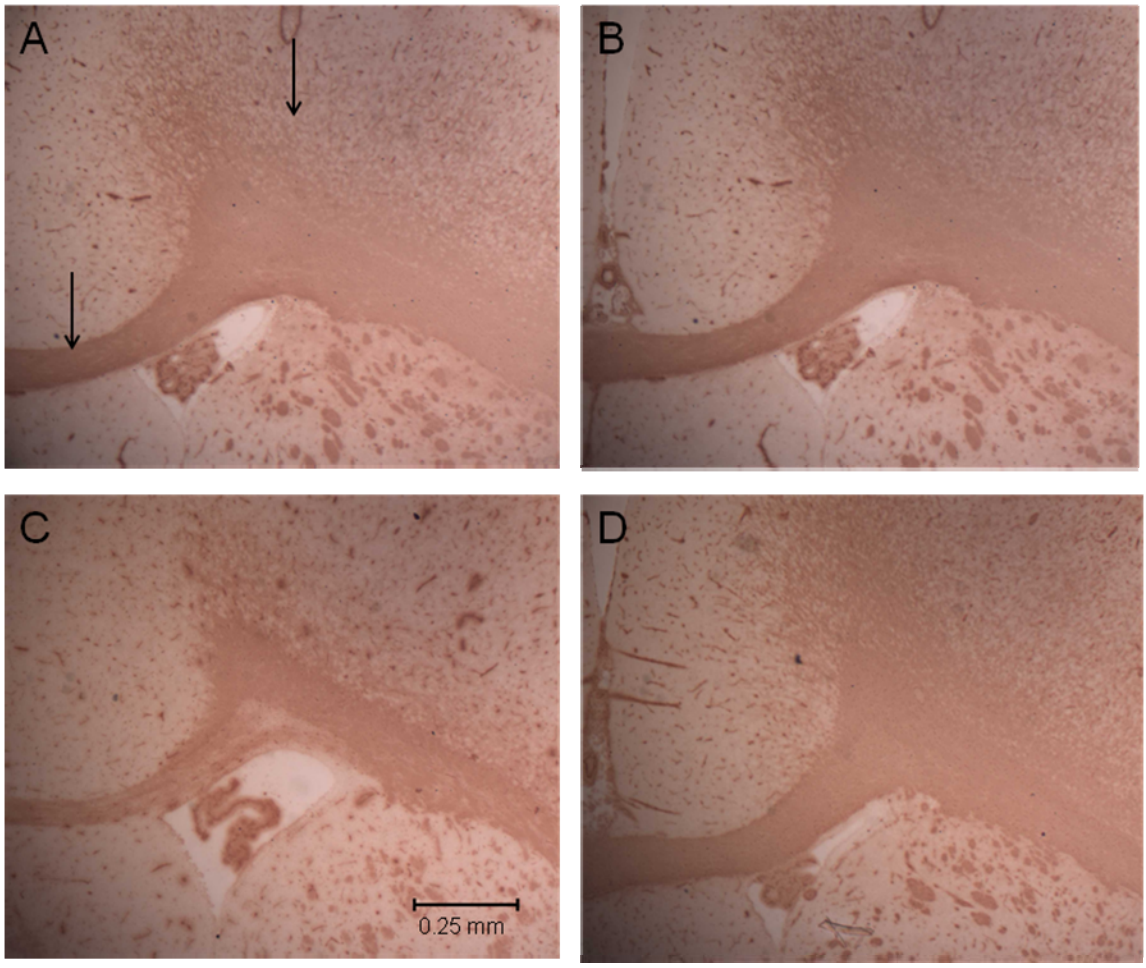


C **D**



Legend: MBP stained posterior brain sections, **A** SHAM, **B** SHAM+B, **C** IUGR, **D** IUGR+B. As with the anterior sections, widespread loss of MBP signal can be seen in the IUGR image, which is visibly improved in the IUGR+B image.

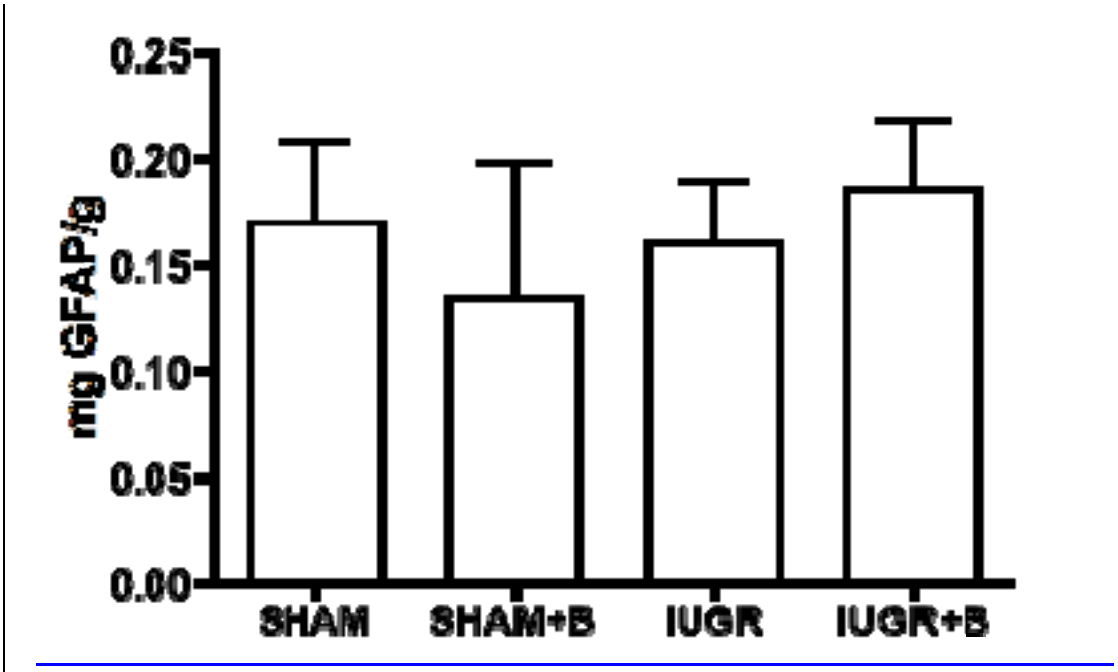
Figure 19



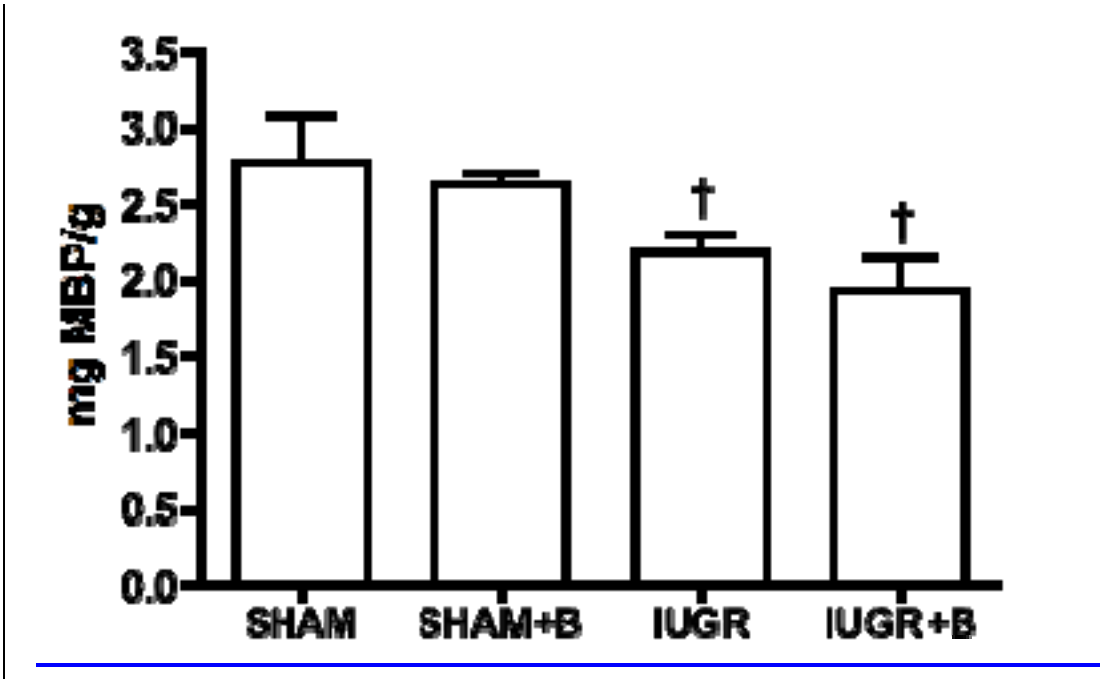
Legend: Photomicrographs show myelin basic protein (MBP) stained images, **A** SHAM, **B** SHAM+B, **C** IUGR, **D** IUGR+B. Close-up anterior brain images were taken at a vantage point to include the right hemisphere ventricle, corpus callosum (cc) and cingular projections. In SHAM animals (A), white matter is densely stained, portraying thick myelinated bundles of the cc and long axonal projections of the cingulum (black arrows). The weak MBP signal observable in the same areas of the IUGR image (C) is typical of our findings and reveals a thinning of the cc and shorter cingular projections. Ventricular dilation typical of the IUGR animals is also visible. The IUGR+B image shows clear improvement over IUGR in all these aforementioned areas.

Figure 20

a) GFAP



b) MBP



Legend: ELISA results for GFAP and MBP

Whole brain samples from each experimental group (n = 6) were evaluated by ELISA. No differences were found for GFAP. For MBP, IUGR and IUGR+B groups showed a significant reduction in signal compared to SHAM controls ($p < 0.0001$ by two-way ANOVA; data is expressed as mean ± SD).

Discussion

Due to the improved survivability rates of premature and growth restricted infants, there is increasing awareness of the associated risk for HI injury to the immature brain. As indicated earlier, this can occur at any time during the perinatal period and can result in a host of neurodevelopmental challenges ranging from learning difficulties to mental retardation and cerebral palsy. Moreover, because of the vulnerable nature of the developing brain to common pharmacologic agents, there is a heightened interest in the development of alternative therapies and strategies for prevention and treatment of perinatal brain injury (Volpe, 2001a; Saliba et al., 2007; Ikonomidou, 2009; Yager et al., 2009).

The purpose of this experiment was to ascertain the effectiveness of a natural health product, broccoli sprouts, as a neuroprotectant/preventative agent in an ante-partum rat model of perinatal HI brain injury. To our knowledge, this study is the first to show that broccoli sprout consumption by rat dams, during pregnancy and for the first two weeks of life of the offspring, prevents/protects against the neurodevelopmental difficulties, both structural and functional, associated with a chronic placental insufficient model of an HI insult to the perinatal rat brain.

Preliminarily it was shown that sulforaphane, when given to pregnant rats through broccoli sprouts in the diet or systemically by ip injection, crosses the placenta into the fetus. In this regard, Dr. Yager collaborated with Dr. Paul Talalay, an international expert on the chemoprotective effects of broccoli sprouts. Measurements of DTC levels (dithiocarbamates) allow a determination of the collective metabolites of

sulforaphane and provide a good measure of the presence and activity of sulforaphane (Fahey et al., 1997; Fahey and Talalay, 1999; Shapiro et al., 2001).

Due to known difficulties in translating research and the importance of clinical relevance for this study, comparisons were made between our model and clinical descriptions of IUGR (Vexler et al., 2006; Cheeran et al., 2009). The BUAL model produced an increase in mortality, based on a significant decrease in litter size; disproportionate growth restriction, as indicated by significant differences in birth weight and Cephalization Index (CI); as well as early developmental delay and abnormal behavioural and neuropathologic development. The model mimics clinical aspects of placental-insufficient IUGR, which predominates in developed countries and is described as failure to reach full growth potential for corresponding gestational age, usually falling below the 10^{th} percentile for birth weight, and having a disproportionate growth pattern, known as brain-sparing (Campbell and Thoms, 1977; Breeze and Lees, 2007; Hui and Challis, 2008). Infants born with IUGR have higher morbidity and mortality and are at increased risk of ante and intra-partum hypoxic-ischemic events (Badawi et al., 1998; Aucott et al., 2004). Recently, several studies have confirmed IUGR of this type to be associated with neuropathologic alterations in hippocampal, cortical and white matter areas of the brain (Samuelsen et al., 2007; Skranes et al., 2007; Lodygensky et al., 2008). Early and late childhood neurodevelopmental difficulties and cognitive delay, such as low IQ and poor executive functioning are also commonly reported with CI at birth found to be a strong indicator of these difficulties (McCarton et al., 1996; Feldman and Eidelman, 2006; Geva et al., 2006a; Leitner et al., 2007b; Leonard et al., 2008).

The early neurologic test battery used in this study was adapted mainly from the seminal work of Fox (1965) who studied 45 litters of mice from birth to adulthood and determined reflex testing of this type to be a useful and reliable indicator of normal central nervous system development. Fox also reported that later periods of development and maturation, (when adult-like responses replace early primitive reflexes) are more critical as behaviour at this time is directly influenced by maturational changes in the brain. Brain development and repair as well as animal behaviour can also be influenced by variables like handling, stress, home-cage environment and maternal factors (Kolb and Gibb, 1991; Liu et al., 2000a; Liu et al., 2000b; Diamond, 2001; Schallert et al., 2003). Animals also improve performance of tests over time because they are in effect "practising" the routine. A researcher comfortable with animals and behaviour testing experience is required to achieve good quality results as animal performance can be unpredictable and may be affected by the researcher's own level of stress. Consistence, patience and a degree of vigilance is necessary to ensure reliability and validity of the test responses. During this study, tests were carried out daily at a similar time in the same quiet location. Care was taken to avoid temperature related behavioural changes or prolonged time away from the dam/home-cage environment. All rats in this study were tested by the same researcher and where possible, were scored by a blind observer. SHAM animal responses within this study were highly consistent with little variability regardless of litter. To our knowledge, this is the first study to report on a battery of long-term behavioural testing for newborn and juvenile rats subjected to this model of intra-uterine ischemia. Previous studies on behaviour of growth restricted animals are limited, but supportive of the findings

reported here (Bassan et al., 2005; Tai et al., 2009). As such, I feel the results of the test battery used to be a reliable indicator of neurologic and maturational factors of development for the rat newborns in this study.

Tests of forelimb and hind limb grasp and hind limb placing were very informative in this model and considered better tests for examining early reflex development than the cliff avoidance, righting or gait tests. The latter tests require physical strength and agility, which does not facilitate the neurologic examination in this model because growth restricted animals are weaker, hindered by their small disproportionate size and lack of fat tissue. For example, while carrying out the righting test, it was observed that IUGR pups that did not need to struggle to right themselves as their tiny body would simply fall to the side and they would turn their legs under. Because the IUGR+B pups were just as physically challenged as the IUGR pups, it would be difficult in these tests to tease out any neurologic benefit of the broccoli sprout supplementation. The cliff avoidance test also requires a degree of physical maturity (strength) to accomplish as well as a combination of reflex and awareness. The top-heavy IUGR animals may fail (fall off the cliff) due to their immature and disproportionate physical development. The pure reflex tests (spinal cord) of grasping and placing provided better discrimination between groups regardless of weight or size.

Our examination of early reflexes and maturation in SHAM and IUGR newborn rats indicated neurological deficits were present in IUGR animals. IUGR rat pups showed significant delay in physical maturation and weight gain as well as poorer performance than sham controls in every test. Bassan et al., (2005) reported similar findings of early reflex delay in an SHR model of IUGR. Supplementation of broccoli

sprouts to pregnant or lactating dams did not affect litter size (mortality), birth weight or weight gain of the offspring, but did influence behaviour and maturation in IUGR animals according to some of the tests. Outcome for IUGR pups of forelimb grasp, hind limb placing, cliff avoidance and posture tests were significantly improved by broccoli sprouts in the diet.

In the forelimb grasp test, broccoli sprout supplementation returned responses to control levels. Forelimb grasp is the earliest test given to the pups in our battery, it is easy to implement and provides a reliable response that is readily recognized by the tester. Early strong reflex tests also have the benefit of naivety of the pup to the testing routine, i.e. any potential confounds due to practice (learning) or handling would be unlikely at this stage. In the cliff avoidance test, SHAM+B and IUGR+B responses were significantly improved and not different from each other. Possibly, coordination is improved in the IUGR+B group, in turn improving their ability to perform this task at an earlier age. Improvement in the SHAM+B group is puzzling as they show no difference from SHAM in any other test in this study. The cliff avoidance response is a reflex, but requires strength and mobility to accomplish. The development and synchronization of neural and musculoskeletal networks involved in successful performance of the cliff avoidance reflex are somehow advanced with broccoli supplementation, the mechanisms of which require research beyond the scope of this study, but may relate to a developmental enhancement of specific motor pathways relevant to reflex pathways, which both communicate via the spinal cord. Hind limb placing (reflex) and posture (maturation) tests both showed improvement in IUGR pups due to broccoli, but not to the level of control animals. The posture test results were surprising because broccoli

sprout supplementation did not significantly affect any other aspect of growth or maturation measured. Although age-appropriate posture was not reached compared to controls, these results are encouraging as the mature posture reflects the achievement of a milestone in brain growth and development, indicating normal adult rat motor control is emerging (Bekoff and Trainer, 1979; Brocard et al., 1999).

Inconsistency in the findings from the early behavioural test battery are expected due to high variability in performance among IUGR animals within both chow and broccoli sprout groups. Notably, the majority of our results gave a significant Levine test, that is, the variances across groups were unequal. This finding attests to the high degree of variability in behaviour and maturational factors observed among IUGR groups compared to SHAM controls. Variability is expected as affected rat pups from our model range in severity of IUGR according to how close a fetus is to the ligated artery, (severely affected fetuses are closest to the ligation) (Figure 21). Many dams delivered stillborn pups and many others did not survive 24 hours. Of the survivors, more severely growth restricted animals sometimes exhibited either limp or hypertonic hind limb/forelimb carriage. This spastic limb observation as well as motor reflex deficits has also been reported in a perinatal rabbit model of placental insufficiency (Derrick et al., 2004; Derrick et al., 2007). These observations indicate that the BUAL model used results in a range of injury, depending on severity of growth restriction. I also noticed a difference in cooperativeness between litters, (i.e. the relative ease with which I could elicit the appropriate test response), which could have been due to differences in temperament between litter dams or time since feeding at the time of behaviour testing, for example. Further questions surround to what degree the effects

of perinatal brain injury may have on ultrasonic vocalizations of rat offspring and in turn on maternal behaviour. Ultrasonic communication of offspring has been shown to influence hormonal response and maternal behaviour of the dam (Saucier et al., 2008). There is also clinical evidence to support influence of maternal behaviour on neurodevelopmental outcome of the infants (Feldman and Eidelman, 2006). For all these reasons, it was important to test a variety of litters and to have at least two litters represented per experimental group. The individual circumstance, severity of injury and outcome of each surviving IUGR newborn in the clinic is also highly variable, therefore the variability among rat littermates in this study is still a reasonable comparison to clinical reports (Bos et al., 2001; Rosenberg, 2008).

A limitation of the early reflex test battery is the subjective nature of the scoring. An objective and blind observer was not possible to achieve as the same experienced tester is required for accuracy and consistency in scoring, but IUGR litters are observably different from controls, due to their small size and lack of pigmentation for their age and were often tested in tandem with SHAM controls through different periods of the study, depending on pregnancy of the rats (Figure 22). In contrast, the open field behaviours were video-monitored and scored by a blind observer. By PD21, the young rats are weaning age and IUGR animals, although often smaller, are difficult to distinguish by direct observation from their SHAM peers. As well, because the BUAL model produces a relatively mild global insult and newborn rats produce few identifiable behaviours until further development, IUGR animals may grow into their deficits as they mature, making any differences due to broccoli sprouts more distinguishable by PD21. The three week old rat's brain has matured at a rapid pace and is comparable to that of

a 1-2 year old human child. Clinical research also indicates measures taken at this stage may be more reliable indicators of long-term outcome than neonatal assessments (Villar et al., 1984; McCarton et al., 1996; Ochiai et al., 2008). For example, the large 10-year prospective study of Leitner et al., (2007) showed somatic catch-up growth at age 2 (and age 9-10) of IUGR children correlated with better neurodevelopmental outcome at age 10. These findings indicate moderate to long-term follow up is required in order to completely assess developmental outcome in terms of IUGR and in our case, to know whether a treatment intervention made a significant difference.

Late behavioural examination with the open field test at PD21 showed hyperactivity in IUGR animals, based on ambulation and head lifting behaviour, a finding that is also consistent with the Bassan et al., (2005) and Tai et al., (2009) studies. Recently, magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) studies have found white matter abnormalities of low birth weight infants persist into adolescence and are coexistent with increased psychiatric symptoms, especially of Attention-Deficit/Hyperactivity Disorder (Indredavik et al., 2005; Skranes et al., 2007). In contrast to the hyperactive behaviour of the IUGR animals, broccoli sprout treated IUGR animals behaved no differently from either SHAM or SHAM+B controls with regard to ambulation and no different from SHAM with regard to head lifts. The open field test is commonly used to observe general rat behaviours like movement. In addition to hyperactivity, I directly observed backward ambulation behaviour that was unique to the IUGR animals as well as different hind-quarter posture, which presented as hunched with less hind limb strength and agility in comparison to SHAMs.

The growth and behavioural assessment of this study provides insight into neurologic developmental consequences of placental insufficiency to the perinatal rat. IUGR rats were significantly developmentally delayed and exhibited abnormal open field behaviour (presenting as hyperactivity), at three weeks of age. Despite no differences in growth or weight, broccoli sprout supplemented IUGR rats showed normal or improved performance in some tests of early development as well as normal open field behaviour, compared to SHAM. These significant findings in neurobehavioural development of IUGR and IUGR+B rats suggest differences may be identifiable in neuropathologic assessments as well.

With regard to neuropathology, both the CA1 and CA3 sectors of the hippocampus in IUGR animals at PD21 had diminished pyramidal cell numbers compared to SHAM animals. Similar results have been found in guinea-pig and sheep models of IUGR (Rees et al., 1998; Mallard et al., 2000). In clinical studies, placental insufficiency has been shown to alter hippocampal development and to be associated with deficits in memory function and spatial orientation (Leitner et al., 2005; Geva et al., 2006b; Lodygensky et al., 2008). Selective vulnerability of the hippocampus and in particular, the CA1 region, to an HI insult is well documented and is postulated to be due to a high concentration of NMDA receptors (NMDAr) on pyramidal cells in the CA1 region (Auer et al., 1989).

NMDA receptors are highly expressed in different brain regions including hippocampal CA1 pyramidal cell membranes where they are comprised of different subunit combinations than neighbouring interneurons, which are less susceptible to excitotoxicity (Ferrer et al., 1995; Cauli et al., 2000). Interneurons also have less

capacity for synaptic plasticity and are less responsive in vitro to exogenous NMDA in terms of current and intracellular Ca^{2+} (Avignone et al., 2005). The enhanced responsiveness of CA1 pyramidal neurons to NMDAr activity reflects the highly expressed NR1 subunit on NMDAr in these cells and may underlie their important role in synaptic plasticity (learning and memory) as well as their vulnerability to overstimulation (Avignone et al., 2005). In the late fetal and early newborn days of the infant rat, during a critical period of synaptogenesis, NMDAr are highly sensitized throughout the brain, due to their essential role in normal development of synaptic neurotransmission (Ikonomidou et al., 1999; Shi et al., 2001). Even transient interruption in NMDAr activity during this time results in widespread apoptosis in the developing rat brain, a reaction shown to be related to loss of synaptic connections and a failure to establish normal connections (Ikonomidou et al., 1999; Olney et al., 2002a). In light of this research, a reduction in CA1 pyramidal neurons resulting from our intra-uterine ischemia model is not surprising. Our data shows broccoli sprout treated IUGR animals had significantly more cells than untreated IUGR in both CA1 and CA3 hippocampal areas and importantly, there was no difference in cell number between SHAM and SHAM+B groups.

Neuropathologic differences were also found between IUGR and SHAM animals in developing white matter areas of the juvenile rat brain (PD21). Both the corpus callosum and cingulate projections were significantly diminished in axonal and myelin development in the IUGR animals, with an accompanying ventricular dilation, not unlike the clinical description of perinatal white matter injury (PWMI) or periventricular leukomalacia, the primary pathologic substrate of cerebral palsy (Baud et al., 2004b;

Back, 2006; Volpe, 2009). Our findings support those of Olivier et al., (2005) who previously reported the deleterious effects of this model on developing white matter areas of the corpus callosum and cingulum and the associated glial response from E21 – PD60. As mentioned previously, recent imaging studies provide clinical evidence that white matter disturbances in growth restricted children predict accompanying executive and cognitive difficulties (Skranes et al., 2007; Skranes et al., 2008; Rose et al., 2009). A plethora of previous clinical and animal research indicates that a hypoxic ischemic insult to the developing brain early in the brain growth spurt period, results in mainly white matter damage and disturbances related to the loss of pre-myelinating oligodendrocytes, which are highly susceptible to degeneration by oxidative stress and resulting inflammation (Husain and Juurlink, 1995; Back et al., 1998; Volpe, 2001b; Back et al., 2002; Inder et al., 2002a; Baud et al., 2004b; Back et al., 2005; Olivier et al., 2005; Riddle et al., 2006; Back et al., 2007; Olivier et al., 2007; Volpe, 2009). Conditions of oxidative stress and inflammation are increased in pregnancies of placental insufficiency, indicating a risk for development of mild PWMI in those infants (Inder et al., 2002b; Inder et al., 2002a; Kumar et al., 2008; Saker et al., 2008). Remarkably, in our study, IUGR animals that received the broccoli sprout treatment had thicker white matter bundles, denser myelination and less ventricular dilation than the untreated IUGR animals. In most cases the IUGR+B group was not different from the SHAM controls.

In further accord with Olivier et al., (2005), this study found a greater amount of reactive (GFAP-expressing) astrocytes in the corpus callosum and cingulate projections of IUGR animals compared with SHAM controls (PD21). Astrocytes are more resistant

to ischemic injury than neurons and oligodendrocytes and act as supportive cells through oxidant scavenging, ion regulation, balancing glutamate concentrations and modulating excitatory synaptic transmission as well as releasing trophic factors (Kirchhoff et al., 2001; Chen and Swanson, 2003). In response to injury, astrocytes increase structural proteins like GFAP, up regulate their trophic release as well as increasing expression of antioxidants like glutathione (Chen and Swanson, 2003). Consistent with our previous findings, astrocytic reactivity was significantly reduced in broccoli sprout supplemented IUGR animals.

Unfortunately, ELISA quantification of both MBP and GFAP provided conflicting results. This could be explained by the fact that while significant differences were seen regionally, the brain insult conferred through BUAL is relatively mild and may not be detectable in whole brain samples. The variability of injury produced by the model could also have added inconsistency to ELISA results as only six samples from each group were assayed. Regional assessment with ELISA and an increase of sample size from each group would likely produce more reliable quantification of protein in the areas of interest here.

It is well accepted that following brain injury, cell death occurs acutely at the primary site of insult and then in a delayed manner to cells in the surrounding area, known as the penumbra in examples of focal stroke (Hamrick and Ferriero, 2003). It is thought that these cells of the penumbra may be the best target for rescue or neuroprotective strategies. In global models such as the BUAL model, the most vulnerable cells will experience acute cell death, e.g. oligodendrocytes because of their stage of development. Unfortunately, mechanisms of delayed cell death in the

developing brain are not fully understood making the implementation of therapeutic strategies difficult. However, it is thought immature cells are more vulnerable to apoptosis, or programmed cell death, i.e. otherwise normal immature cells are programmed to delete themselves if for any reason they are unsuccessful in establishing synaptic connections (Ikonomidou, 2009). Many eloquent studies from the Laboratory of Dr. J.W. Olney have not only distinguished the acute excitotoxic from delayed apoptotic cell death, but have also shown evidence of delayed apoptosis occurring in a temporospatial progressive manner in both traumatic and drug-induced brain injury (Olney et al., 2002b; Young et al., 2004; Bayly et al., 2006). In traumatic injury, patterns of evolving circuitry-dependent delayed cell death were reported. Not only were many cells degenerated at the site of impact, but delayed cell death occurred in specific sites distant from the site of impact. It was determined that enough local fibers of the corpus callosum/cingulum bundle were damaged to destroy communication channels to remote sites, causing those neurons to undergo apoptosis, thereby losing more connections, which in turn caused further cell death, like a domino effect. The authors postulated that substantial loss of communication channels determines whether a developing neuron will commit suicide (Bayly et al., 2006).

Recent evidence suggests that delayed cell death in perinatal HI also evolves in a predictable pattern based on neural circuitry. Stone et al., (2008) used a mouse model of neonatal HI (PD7) with DTI imaging and neuropathology to show that subsequent neurodegeneration (in the hippocampal circuit) was prolonged (up to PD42), progressive and sequential. Susceptibility of downstream areas depended on neural connectivity with the initial site of injury (Stone et al., 2008). Our current study

shows IUGR results in a significant deficiency of the corpus callosum/cingulum bundle. This major fiber pathway is the communication channel for many neuronal inputs and outputs. Major alterations in development of this fiber tract could result in deletion of cells that did not receive expected synaptic inputs or did not successfully transmit outputs. Furthermore, recent research from the Laboratory of Dr. R.H. Lane has not only described increased apoptosis, but also sex-specific epigenetic alterations of the BUAL model in IUGR rats. These authors have found non-functional circuitry of the apoptotic regulator p53, modified chromatin structure, which causes persistent transcriptional alterations, changes in cerebral mitochondrial gene expression and more recently, altered NMDAr subunit composition in IUGR hippocampal regions (Lane et al., 2000; Lane et al., 2001; Ke et al., 2005; Ke et al., 2006; Schober et al., 2009). Chronic placental insufficiency confers a mild insult to the fetal brain, but one that evolves with development. In any perinatal brain insult, the ensuing interplay between destructive consequences of injury, effects of repair and plasticity and the continued maturation of nervous tissue and systems will together determine outcome. This multifaceted condition has the potential to result in a vast spectrum of developmental disturbances or alterations that shape structure and function of each affected individual.

Placental insufficiency induced by BUAL in the rat, models clinical perinatal HI injury that can occur in utero, in the preterm developing brain when pre-myelinating oligodendrocytes are most susceptible to oxidative stress, resulting in primarily white matter damage. The resultant injury from exposure to HI depends on the developmental stage of the brain at that time and many other factors as discussed, but the fundamental mechanism underlying HI injury is constant. A reduction in blood flow,

accompanied by the curtailment of oxygen and glucose delivery leads to a cascade of complex cellular events including, energy failure, over activation of glutamatergic neurotransmission (excitotoxicity), a perpetuation of cell edema and in turn, a rapid elevation of intracellular calcium (Rothman and Olney, 1986). Resulting cell damage enhances free-radical or reactive oxygen species (ROS -unstable molecules containing an unpaired valence electron) production, which play an important role in the pathogenesis of post-ischemic injury by destroying mitochondrial and cell membranes, causing a release of pro-apoptotic effectors as well as activating NFκB, the transcriptional factor that up regulates the inflammatory response, which contributes further to overall damage (Juurlink, 2001; Northington et al., 2001; Jantzie et al., 2005; Blomgren and Hagberg, 2006).

Unfortunately, the perinatal brain is particularly susceptible to oxidative stress due to a high concentration of fatty acids, high rate of oxygen consumption and immature functioning of antioxidant enzymes such as superoxide dismutases (Halliwell, 1992; Thorburne and Juurlink, 1996; Fullerton et al., 1998; Ferriero, 2001; Baud et al., 2004a; Folkerth et al., 2004). The known vulnerabilities of the immature brain has led experts to suggest the use of clinically safe free radical scavengers to be one possible way to treat H/I injury in the newborn (Volpe, 2001a; Back, 2006). There is evidence to suggest antioxidants can provide neuroprotective benefit. In this regard, over expression of the endogenous antioxidant, glutathione peroxidase provided protection to neurons in culture and in knock-out mice (Wang et al., 2003; McLean et al., 2005). Moreover, both grape seed oil and pomegranate polyphenols have proven protective in animal models of HI (Feng et al., 2007; West et al., 2007).

Recently, there has been an explosion of research on broccoli, in particular its constituent, sulforaphane (Figure 23). Sulforaphane gained attention as a chemoprotective compound due to the pioneering efforts of Dr, Paul Talalay and has also been shown to have a profound effect on reducing hypertension and inflammation in the cardiovascular system of SHR rats (Juurlink, 2001). With respect to the brain, sulforaphane has recently been reported to protect the blood brain barrier and improve cognitive outcome after traumatic brain injury, to modulate neuro-inflammation, to reduce infarct volume following MCA occlusion and to improve cell counts and neurologic deficit score in a model of intracerebral hemorrhage (Zhao et al., 2005; Zhao et al., 2006; Zhao et al., 2007; Innamorato et al., 2008; Dash et al., 2009).

The particular therapeutic benefit of broccoli sprouts is attributable to its high content of glucoraphanin, which is the glucosinolate precursor to the isothiocyanate sulforaphane. Isothiocyanates, increase expression of the transcription factor, Nrf2 (nuclear factor E2-related factor 2), which binds to the antioxidant response element (ARE) and induces Phase II enzymes (eg. glutathione transferases, quinone reductase and heme oxygenase) as well as other cytoprotective proteins (eg. thioredoxin, glutathione peroxidase and superoxide dismutases), which are collectively important in endogenous scavenging of ROS (Fahey and Talalay, 1999; Warner et al., 2004; Dinkova-Kostova and Talalay, 2008). Phase II enzyme induction also enhances the activity of γ-glutamylcysteine synthetase, the rate-limiting enzyme involved in the synthesis of the endogenous antioxidant glutathione, known to play a central role in oxidant scavenging and to be a major antioxidant of the brain (Juurlink, 1999; Aoyama et al., 2008). In fact, it has been shown that glutathione (GSH) depletion alone is

sufficient to cause oxidative stress-induced death in immature oligodendrocytes (Yonezawa et al., 1996; Back et al., 1998). Alternatively, small increases in GSH prevent cellular oxidative stress of oligodendrocytes (Thorburne and Juurlink, 1996). There is also mounting evidence that GSH depletion is a contributing event in the activation of signalling pathways that lead to the progression of apoptosis (Franco and Cidlowski, 2009).

Under physiologic conditions, production and elimination of strong oxidants in the brain is a normal necessary process that proceeds without incident provided there is enough available substrate to neutralize reactive species. The brain is a major producer of strong oxidants, comprising only about 2% of total body weight, but using approximately 20% of total O_2 consumption (Aoyama et al., 2008). Mitochondrial respiration of cells produces the superoxide radical (O_2^-), which is eliminated through conversion by superoxide dismutase to hydrogen peroxide (H_2O_2), which is then reduced to O_2 and H_2O by glutathione peroxidase or catalase (Chance et al., 1979; Juurlink, 2001; Dringen et al., 2005; Aoyama et al., 2008). This clearance is of major importance to the detoxification of cells and proceeds in a very efficient manner in all cells of the CNS (Dringen et al., 2005). Unfortunately, if enzyme activity is burdened as with response to H/I injury, H_2O_2 can react with iron to produce hydroxyl radicals (OH^-) and O_2^- can react with nitric oxide (NO) to produce the toxic peroxynitrite ($ONOO^-$) radical, together these reactive species cause lipid peroxidation, DNA damage and inactivation of mitochondrial enzymes, leading to energy failure (Aoyama et al., 2008).

Glutathione, in its reduced state (GSH), is an obligatory participant in the reduction of H_2O_2 by glutathione peroxidase (GPx) and is converted to its oxidized form

(GSSG) during the reaction. Oxidized glutathione is readily converted to its reduced form in a subsequent reaction catalyzed by glutathione reductase (GR) and NADPH and is then reused as a GPx substrate (Dinkova-Kostova and Talalay, 2008). GSH participates in many other direct and indirect cellular antioxidant reactions including non-enzymatic reduction of O_2^-, NO, OH^- and $ONOO^-$ and is a cofactor in xenobiotic metabolism, exporting various toxins from the cell (Aoyama et al., 2008) (Figures 24-25). Levels of GSH are known to fluctuate during oxidative stress and a common measure of cellular redox environment is the relative ratio of GSH to GSSG (Juurlink, 2001; Franco and Cidlowski, 2009). During the injury response, endogenous cellular antioxidants like GSH and glutathione peroxidase are depleted causing the imbalance between production and reduction of strong oxidants, which can take up to 72 hours to be restored to normal concentrations (Namba et al., 2001; Warner et al., 2004). Enzyme induction by the sulforaphane – mediated Nrf2 driven response is long-lasting and includes all the important enzymes in GSH synthesis and metabolism (Fahey et al., 1997) (Figures 3 & 4).

There has been speculation in the literature that the developing brain is deficient in antioxidant capabilities. Oligodendroglial precursors in particular, show a developmental lag in SOD expression, presumably making infants more susceptible to oxidative-mediated white matter injury (Folkerth et al., 2004; Folkerth, 2006). However, developing neurons and glia contain all the defence mechanisms of mature brain cells and in some developmental stages, actually over-express antioxidant enzymes and GSH compared to mature cells, which may account for some controversy in the literature (Fullerton et al., 1998; Dringen, 2000; Folkerth, 2006; Sun et al., 2006). A

more correct interpretation may be that the immature brain lacks efficient compensatory abilities upon encountering oxidative stress. For example, studies have shown over expression of copper/zinc superoxide dismutase (SOD1) in neonatal mice exposed to HI to result in a paradoxical increase in damage, possibly due to the depletion of GPx and catalase during the injury response (Ditelberg et al., 1996; Fullerton et al., 1998). A similar study in the adult however, showed SOD1 to be protective and caused a compensatory increase in catalase activity that did not occur in the neonate (Przedborski et al., 1992). Other studies have also shown the contrasting neuroprotection of SOD1 over expression in adult models of ischemia as well (Chan et al., 1994; Yang et al., 1994; Francis et al., 1997). These results indicate that balance between these endogenous enzymes may be more important in the perinatal brain than their absolute concentrations (Fullerton et al., 1998).

Complete understanding of the complexity of these aspects of cell metabolism cannot be ascertained by whole brain measurements of enzyme activity. Many studies have focused in vitro on various brain cell types and stages of development and have shown although cells similarly express these enzymes and pathways, they show discrepancies with respect to concentration levels, substrate affinities and inherent resistance to oxidative stress, a common underlying reason being differential ability to express GSH (Dringen, 2000; Dringen et al., 2000; Hirrlinger et al., 2000; Sun et al., 2006). As mentioned previously, Back et al., (1998) showed GSH depletion alone was sufficient to cause oligodendroglial cell death. Similarly, up-regulation of SOD activity was shown to be protective to developing oligodendrocytes in culture when exposed to mild oxidative stress, however not with severe cysteine deprivation (or limited GSH

synthesis) (Baud et al., 2004a). Additionally, Murphy et al., (1990) showed immature cortical neurons succumb to glutamate toxicity even prior to NMDAr development (Murphy et al., 1989; Murphy et al., 1990). The same researcher subsequently showed protection of this phenomenon by up regulation of the Phase II enxyme, NADPH:quinone reductase (Murphy et al., 1991). Since then, many studies have shown an over abundance of glutamate inhibits normal functioning of the glutamate/cysteine exchange mechanism, which facilitates cysteine uptake by the cell (Murphy and Baraban, 1990; Murphy et al., 1990; Sagara et al., 1993a; Shih et al., 2006; La Bella et al., 2007; Aoyama et al., 2008). Cysteine deprivation leads to diminished GSH levels, increased ROS and eventually, apoptosis (Franco and Cidlowski, 2009). In this regard, over-expression of SOD or GPx may be insufficient to provide protection without a concomitant increase in GSH production or cysteine availability.

Further complexity is added from studies that show astrocytes to be protective of neurons in culture, contain higher concentrations of GSH and provide cysteine to neurons for GSH synthesis (Sagara et al., 1993b; Kirchhoff et al., 2001; Dringen et al., 2005; Shih et al., 2006). In fact, over-expression of the cysteine/glutamate transporter in cultured astrocytes was shown to provide complete neuroprotection to neighboring neurons, due to the increase in astrocytic concentrations of GSH (Shih et al., 2006). Sulforaphane has been reported to protect cultured astrocytes from O_2 and glucose deprivation and to preferentially activate the Nrf2 response in astrocytes over neurons (Kraft et al., 2004; Danilov et al., 2009). Intuitively, not only are broccoli sprouts a good dietary source of cysteine, but the cysteine/glutamate transporter is also up regulated during the Nrf2 response (Shih et al., 2006). With direct relevance to our study,

Schober et al., (2009) reported significant increases in brain levels of cysteine, adenosine and cysteinylglycine as well as a decrease in GSH (non-significant) after BUAL on P0, indicating an endogenous effort to protect against oxidative stress. Presumably, induction of Phase II detoxifying enzymes by sulforaphane in our model of perinatal H/I injury, activated Nrf2, thereby greatly enhancing antioxidant capabilities of cells, which decreased ROS and the pro-inflammatory response and ultimately made a significant contribution to cellular defences, improving neurologic outcome of IUGR rat offspring. Future studies should determine whether broccoli sprout supplementation can provide long-term neuroprotection (to adulthood) in this model and other models of perinatal H/I injury and whether direct sulforaphane administration provides comparable protection. As well, the inflammatory component of this injury, although understood, was not investigated in this study, but would be a worthwhile area of future research as well. Finally, research directed at discovery of *in vivo* cellular metabolic activity responding to sulforaphane ingestion or Nrf2 activation during the injury response, would provide greater insight into mechanisms of oxidative stress, apoptotic pathways and specific cellular defence mechanisms not yet elucidated.

In summary, during the perinatal period, the developing brain is inherently susceptible to injury for an amalgamation of reasons. Immature and/or specialized systems of angiogenesis, glia and neurogenesis, cell metabolism, cell signalling and endogenous enzyme activity are ill-equipped to withstand any reduction in energy or homeostatic imbalance at this stage. Synaptogenesis, or neural communication development is experiencing a critical stage where high concentrations of NMDA

receptors, which are crucial for synaptic development, are specialized during this period and unfortunately, highly sensitive to hypo or hyper stimulation. An interruption in this normal synaptic development due to an H/I or drug-induced event affects multiple systems, causing faulty connections, cell signalling confusion/failure and subsequent neurodegeneration. The effect of any one insult also depends on the region/type of injury and the specific stage of development all systems are functioning in during the event. Unfortunately, implementing therapies for the perinatal brain is analogous to aiming at a moving target. Conventional therapies developed for therapeutic use in Pediatric medicine such as caffeine and anti-convulsants as well as agents developed to reduce damage in adult brain ischemia have proven harmful to the immature brain. Clearly, even transient influences that strain or cause deviations to intricate mechanisms of homeostasis in the perinatal brain may set developmental trajectories off course.

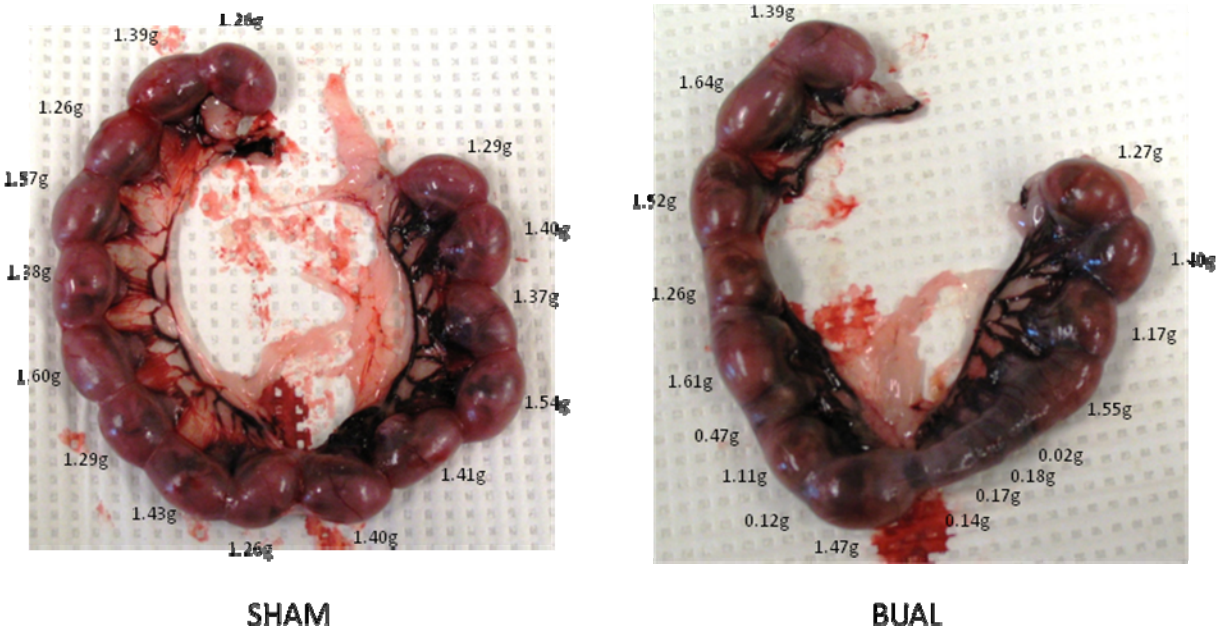
The ability to provide treatment to mother and fetus is hindered by ethical barriers of safety. Unfortunately, without knowing which pregnancies are at risk and when treatment should be implemented, it is impossible to develop safe and effective therapies. Complementary and Alternative Medicines like natural health products provide an alternative to conventional drug therapies, which are unlikely to ever be approved for use in pregnant women and tend to be administered as “rescue” therapies for the fetus or newborn already in jeopardy. The utilization of a safe preventative option is a better approach to combating perinatal brain injury. Broccoli sprouts are an excellent dietary source of sulforaphane, and a naturally occurring substance that is widely consumed around the globe with no reported safety or tolerance concerns. A

clinical phase I study on safety, tolerance and metabolism of repeated oral dosing of broccoli sprout glucosinolates and isothiocyanates found no associated significant toxicities (Shapiro et al., 2006).

In this study it was shown providing broccoli sprouts to pregnant rats in the last trimester of gestation and first two weeks of life of the offspring, lessened neurologic effects of placental insufficiency. These findings suggest a novel approach to preventing developmental disabilities associated with perinatal brain injury that can be provided safely to mother and fetus. This approach is not without precedent as folate supplementation during pregnancy as well as dietary fortification for prevention of neural tube defects has proven very successful over the years (Czeizel and Dudas, 1992; Bol et al., 2006). A recent study by Ward and Beachy (2003) published data pertaining to the increasing survival of premature infants. Between 1981 and 2000, the survival of infants 22-24 weeks gestational age increased from zero to between 50-75% (Ward and Beachy, 2003). If we are to continue to develop technologies to provide life support for premature and growth restricted infants then we must endeavour to seek with equal diligence, the knowledge leading to the development of strategies that will provide quality of life as well.

Discussion Figures

Figure 21



Legend: Fetal impact of bilateral uterine artery ligation

BUAL surgery was performed on E15 of gestation (Sprague-Dawley rat strain). On E19 caesarean sections were performed and each fetus was weighed and compared to gestation-matched controls. These images illustrate the variability in fetal impact of the BUAL model as the fetuses closest to the ligation (inferior uterine arteries) are affected most severely.

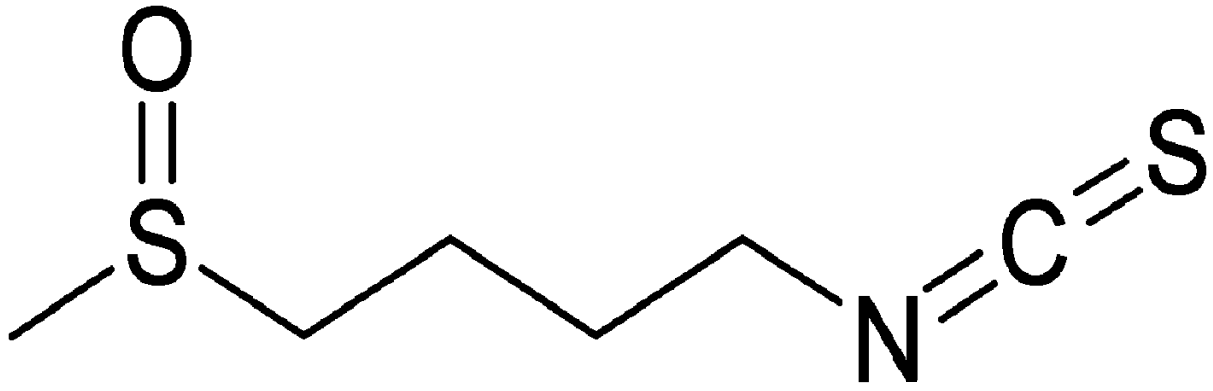
Figure 22

PD7 **PD21**

Legend:

As indicated by the picture of these rat pups at PD7, IUGR animals (on the left) are observably smaller after a week of life and are slower to develop a normal phenotype, eg. pigmentation, than their SHAM peers (on the right). Although a difference in size is still seen at PD21, IUGR animals at this stage are more difficult to distinguish by direct observation alone.

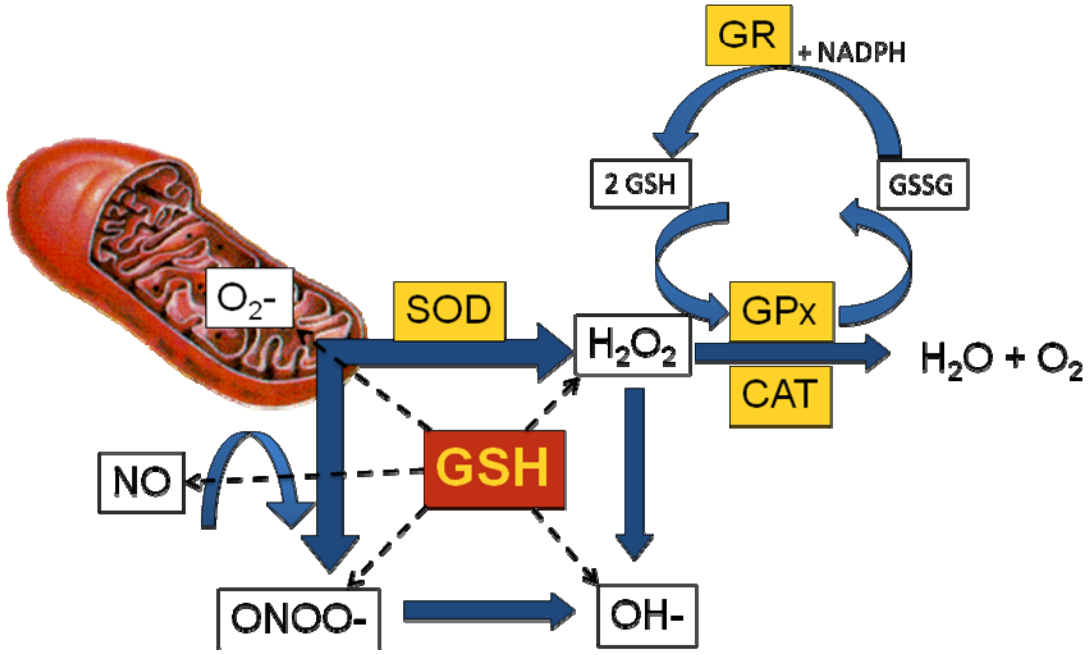
Figure 23



Legend: Sulforaphane chemical structure

The ⁻N=C=S group is highly electrophilic and reacts readily with O, N and S based nucleophiles. Sulforaphane is not known to participate in direct reduction or oxidation reactions and does not have any pro-oxidant activities as some direct acting antioxidants.

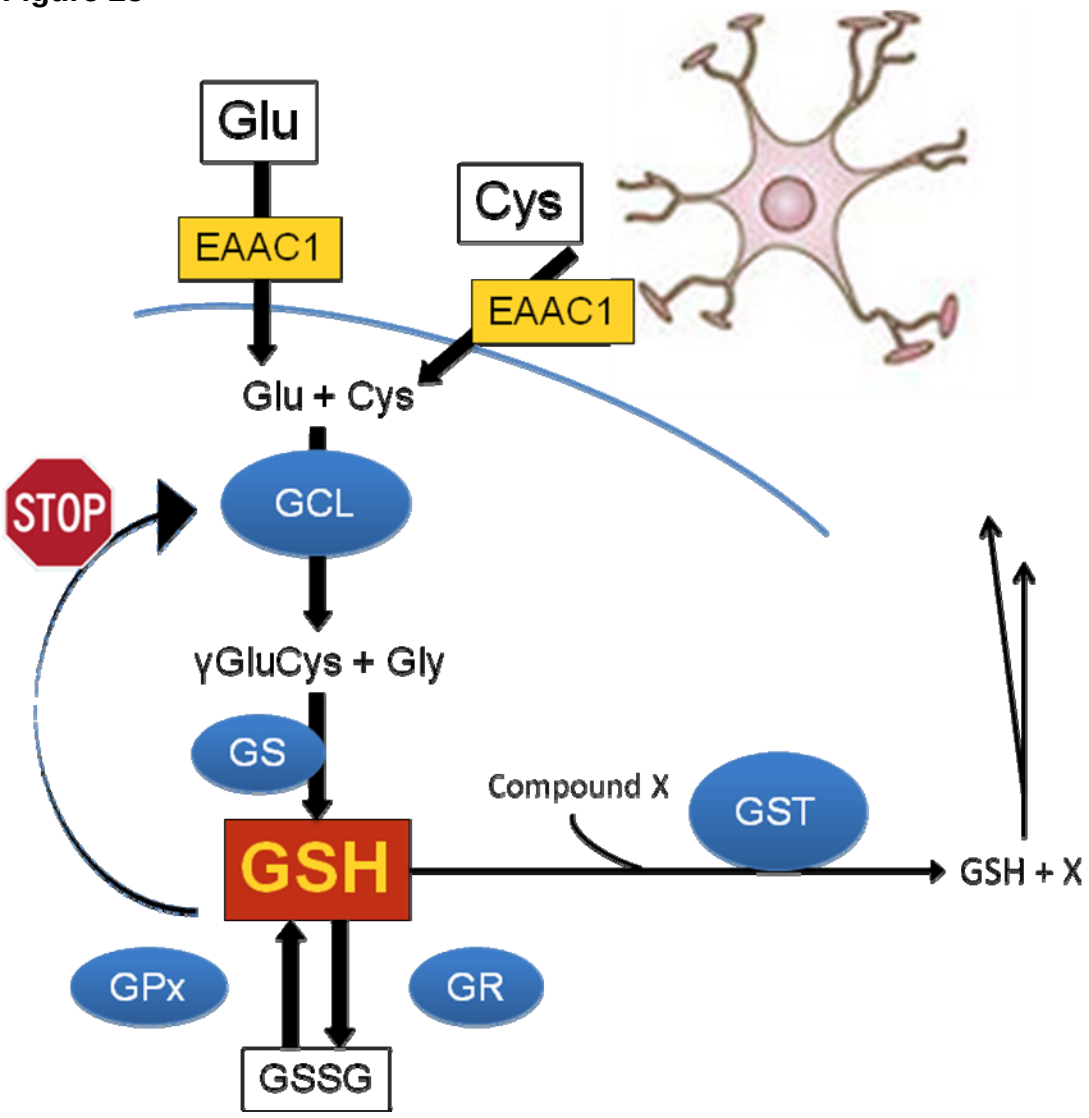
Figure 24



Legend: Glutathione: the major direct endogenous antioxidant of the brain

Mitochondrial respiration produces substantial amounts of superoxide anions that are reduced by superoxide dismutase to hydrogen peroxide, which in turn, is reduced further by glutathione peroxidase. Glutathione is an obligatory participant in the breakdown of H_2O_2 and other peroxides by glutathione peroxidase, producing glutathione disulfide, which is readily converted back to its reduced form by glutathione reductase and NADPH in a continuous cycle. The cartoon further illustrates the multifunctional non-enzymatic antioxidant capabilities of glutathione (dotted arrows). Abbreviations: O2- : superoxide; NO: nitric oxide; ONOO- : peroxynitrite; OH- : hydroxyl radical; H_2O_2 : hydrogen peroxide; GSH: (reduced) glutathione; GSSG: oxidized glutathione; SOD: superoxide dismutase; GPx: glutathione peroxidase; CAT: catalase; GSSG: glutathione disulfide; GR: glutathione reductase

Figure 25



Legend: Glutathione Synthesis

Glutathione is synthesized in the neuronal cytoplasm from glutamate, cysteine and glycine in two enzymatic steps. The rate-limiting substrate for neuronal glutathione production is cysteine, provided through export from astrocytes via excitatory amino acid carriers. The first and rate-limiting step of synthesis is catalyzed by γ-glutamylcysteine synthetase (aka GCL), which is regulated via feedback inhibition of GCL by glutathione. Upon completion, glutathione is available for redox reactions or

can react with various xenobiotic compounds via glutathione-*S*-transferase to form disulfides, which are then exported from the cell. Abbreviations: EACC: excitatory amino acid carrier; GCL: γ-glutamylcysteine synthetase; GSH: glutathione; GST: glutathione-*S*-transferase;

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