An investigation of the effect of *Bifidobacterium infantis* on hippocampal interleukin-6 levels in a rodent model of hypoxia-ischemia following preterm birth

by

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Abstract

Inflammation has modulatory effects on the brain, particularly during development. These plastic changes can hold severe functional consequences. Perinatal hypoxia-ischemia (HI)-induced inflammation can result in cerebral palsy and cognitive impairment. In an attempt to reduce inflammation in the brain, we assessed the probiotic *Bifidobacterium (B.) infantis* as an HI intervention, using a rat model. Rat pups, developmentally equivalent to preterm infants, were exposed to chronic hypoxia from postnatal (PND) 3 –PND 10. Inflammation was assessed through hippocampal concentrations of the cytokine interleukin-6 (IL-6). Tissue was collected from pups on PND 10 and analyzed via enzyme-linked immunosorbent assay (ELISA). Results showed lower IL-6 concentrations in hypoxic groups , regardless of *B. infantis* administration. Qualitative observations suggested poor gut health in association with hypoxia and probiotic exposure. These preliminary findings support the chronic hypoxia exposure model of HI and suggest the association with IL-6 and HI events is less straightforward than expected.

Chapter 1: Brain plasticity

Brain plasticity references the structural and operational malleability of the brain as a function of experience. Until mid-20th century, brain change was considered limited to the initial organization associated with early development. Continued refinement in experimentation allowed for demonstrations of maintained malleability across the lifespan, establishing a biological basis for behavioural differences, such as memories, developed later in life. Brain function is now viewed as reliant on the ongoing interplay between biological mandate and experiential influence. A pivotal shift towards this dynamic conceptualization of the brain came from the household of Donald Hebb, who famously brought home laboratory rats to explore his house and play with his children (reviewed by Brown & Milner, 2003). These rats, when returned to the laboratory as adults for behavioural testing, exhibited superior cognitive performance relative to rats reared in the traditional cage setting (Hebb, 1947). From this, Hebb inferred some factor of the experience held by his pet rats had impacted their brain function. Hebb's theories, based on behavioural evidence and intuition, provided a model for future research. Over time, the conceptualization of plasticity evolved from a finite process to a continuous phenomenon, necessary for biological and behavioural individuality.

Change is inherent to early development, as the brain grows from single cells according to a genetic template. Brain development is initiated with the production of neural progenitor cells from embryonic tissue. These cells are the constituents for every component of the central nervous system (CNS), and they migrate from their position in the embryo according to a predetermined genetic plan. Neural progenitor cells form the neural tube, which produces the neuroepithelium, later dividing into the telencephalon, diencephalon, metencephalon, myelencephalon, and the mesencephalon. Each subdivision is responsible for the creation of

specific brain structures. The next phase is the production of neurons, with developmental courses dependent on the structure in question. Neurons then continuously migrate, resulting in the 6-layer organization of the neocortex. This assembly is followed by neuron differentiation (with distinctions based on layer) and dendritic arborisation, as the cell pursues connection with other cells. Unnecessary cells and cell associations are eliminated via apoptosis and pruning. Developmental expectations of the brain are only met when change is both additive and purposefully destructive.

In contrast to developmental plasticity, a blueprint does not dictate adaptive plasticity. Plasticity that occurs outside of innate developmental processes is responsive, producing change as a consequence of experiential circumstances. These changes are evident from both a gross anatomical and microscopic perspective. Anatomical change includes the loss of cells (atrophy), the addition of cells (neurogenesis and/or gliogenesis), as well as the modification of cell roles. Microscopic change ranges from alterations in dendritic structures to modification of chemical messenger gradients implicated in synaptic communication. These biological differences are revealed in function, from differences in cognitive ability to variance in motor skills. Through large and small modifications, the brain is adjusted for the specific environment in which it will function.

There are many factors that induce brain change. Although plasticity serves many purposes involved with healthy function, both inside and outside of early development, adverse outcomes arise following certain experiential stimuli. The number of factors linked to negative consequences is continuously growing. In order to best understand the functional consequences of change following a specific event, consideration should be given to the previously established possibilities for change, the key principles of plasticity, and the current comprehension of the

mechanisms of change following the specified event. Further exploration of the structural and functional outcomes of brain change will contribute to a broader understanding of plasticity, including both the favourable and unfavourable effects.

1.1 Established processes of change

Although Hebb often receives credit for being the father of neuroscience research, much of his contributions were based on theory rather than anatomical or physiological evidence. Without the means to explain the mechanisms of electrical communication between cells in their entirety, Hebb proposed that repetitive experience influenced the relationship between cells through some increase in communication efficiency (Hebb, 1949). Specifically, the Hebb postulate (Hebb, 1949) states "when an axon of cell A is near enough to excite a cell B and repeatedly and persistently takes place in firing it, some growth or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased." The significance of this suggestion lay in cells possessing the potential to be trained to interact with one another differently, consequently forming novel cell relationships. Although Hebb's ideas regarding the synapse and the brain's need for plastic change were only theoretical, his work would become foundational to modern literature on the function of the brain.

The first research group to substantiate physical changes in the brain following experience was Rosenzweig and colleagues. They ran multiple experiments on the structural consequences of environmental enrichment in the developed rodent brain, as comparable mechanisms between the human and rodent brain allow for inference regarding human circumstances. Enrichment in the human condition is associated with increased sensory stimulation, replicated in the rodent through larger cages, the presence of toys, increased handling, and variance in water bottle and food locations. One of the first measures of structural

change in response to environmental enrichment was cerebral cortex weight, found to be positively correlated with the environmental complexity an animal had been exposed to (Rosenzweig et al., 1962). This study was followed by research providing explanations for the increase in weight, including demonstrations that environmental enrichment correlated with the proliferation of glial cells (Altman & Das, 1964) and increased cell size (Diamond, Linder, & Raymond, 1967). In the visual system of cats, Hubel and Wiesel (1963) demonstrated limiting experience could also influence cell size. This effect was shown by suturing the eyelids of kittens prior to eye opening and measuring the reduced cell size of neurons in the lateral geniculate nucleus (LGN) 3 months later (Hubel & Wiesel, 1963). These studies provided conclusive evidence that the brain was anatomically changed by experiential influences.

Anatomical change, however, refers to both shape and structure. Once shape changes were evident in the brain following experience, Hubel and Wiesel sought to identify whether the functional structure of the brain could be reorganized. The two colleagues, considered the fathers of plastic physiology, initially examined whether the functional role of individual cells could be alternated. Hubel and Wiesel (1965a) found most cells in the cat LGN demonstrated an innate role, preferentially relaying sensory input from one eye. There were some cells, however, lacking natural eye specificity. Following visual sensory deprivation to one eye in kittens, the cells demonstrating non-specificity would shift to unitarily responding to the non-deprived eye, reflecting the new needlessness for response to the inactive eye. When monocular deprivation was removed after three months, the cells maintained their preference and vision remained impaired (Wiesel & Hubel, 1965b). These results indicated cell function was malleable in the visual cortex, and individual cells roles could be manipulated permanently.

Maintaining consideration of Hebb's emphasis on cell relationships, research turned to exploring whether cell networks could be directed to change roles. Merzenich (1983) chose to explore outside of the visual cortex, examining the cell networks responsible for sensory interpretation of the hand. Specifically, he looked at area 3b of the primary somatosensory cortex, which represents three different nerves in the hand: the median, ulnar and radial. When the median nerve was severed, thereby no longer providing sensory stimulation, the cells in its representative cortical area began to collectively fire for the two remaining nerves. Through this, Merzenich's research group demonstrated removing sensory stimulation produced functional reorganization of a cell assembly. This malleability had multiple adaptive purposes, as it was also demonstrated following the addition of excessive sensory stimulation. Merzenich's research group found larger cortical representations of the hand in monkeys who had received repeated, specific motor training (Jenkins et al., 1990). Although individual layers of the same cortical region could reorganize independently following experience (Withers & Greenough, 1989), networks of cells demonstrated orchestrated change. Evidently, entire cell assemblies could be manipulated following experience.

Further advancements in technology enabled investigators to explore the cellular factors involved with changes in cell relationships. Prior to the opportunity to explore microscopic change, the hippocampus had garnered interest from the neuroscience community due to its implication in learning and memory (Scoville & Milner, 1957), primary behavioural examples of plasticity. Initial cellular work involving the hippocampus entailed determining the relevance of dendritic structure to plasticity, as dendrites support the majority of a neuron's synapses. Greenough and Volkmar completed the first studies looking at dendritic plasticity, or the modulation of dendritic structure, in response to experience. The authors found up to 20%

increases in dendritic fields in animals reared in complex environments relative to controls (Greenough & Volkmar, 1973). Enhanced cell connection from environmental enrichment was also demonstrated with increases in synapse concentrations (Globus et al., 1973) and spine ratios per neuron in adult rats (Turner & Greenough, 1985). These studies identified the importance of cell connection in plastic change.

With attention now directed at the synapse, Bliss and Lømo (1973) were the first to provide evidence for Hebb's inter-cellular theory. They developed a model in which to explore the mechanisms involved with changes in cell communication. Using rabbits, the investigators determined repetitive electrical stimulation of the dentate area of the hippocampal formation resulted in long-term post-synaptic excitability. It appeared the efficiency of signal transfer between the pre-synaptic and post-synaptic cell populations could be increased by repetitive exposure to serial pulses, an effect they originally called "long-lasting potentiation" (LTP). The complementary process to LTP is long-term depression (LTD), where the efficiency of inter-cellular communication is reduced following sensory stimulation, or lack thereof. Both synaptic phenomena are associated with the microscopic changes involved with plasticity.

On the heels of Bliss and Lømo's (1973) novel findings regarding cell communication, Stent (1973) considered the underlying changes involved with altered synaptic efficiency. He proposed postsynaptic receptors not directly involved with a surge or drop in postsynaptic activity would be eradicated due to lack of need, thereby fine-tuning a cell's sensitivity to a given stimulus. LTP would then be made possible through cell populations synchronizing their specificity tuning. Years later, Singer (1985) hypothesized synaptic change would also need a certain activation threshold passed on the post-synaptic side, given the link between excitation and increased communication. Singer was most interested in the relationship between voltage-

dependent calcium channels and this proposed threshold, as he could demonstrate a correlation between calcium fluxes and cell response (Geiger & Singer, 1986). Taken together, the relevance of microscopic impact on the synapse has been shown, both in structure and neurochemical composition.

Since Hebb's theory, an understanding of the multiple facets of change involved with plasticity has developed. The perspective held by the scientific community on the malleability of the brain changed drastically, from contingent on developmental timelines to continuous across the lifespan. Inter-cellular communication has proven pivotal to plastic change. What is now known regarding plasticity provides a foundation for future studies investigating unknown circumstances and mechanisms of change.

1.2 Key principles of plasticity

Brain plasticity has been shown to follow general principles, providing a framework with which to understand all circumstances of brain change.

Plasticity is activity-dependent

From birth on, there are two types of plasticity: *experience-expectant* and *experience-dependent* (reviewed by Kolb & Gibb, 2015). Experience-expectant plasticity is reliant on signals from the environment to shape the direction of development. Experience-expectant plasticity is also referred to as developmental plasticity. An example of this form of change is seen in audition. Infants receiving limited auditory stimulation, such as in the case of chronic ear infections or congenital hearing loss, will have underdeveloped auditory perception and later issues with speech production (Ponton et al. 1996). These behavioural effects correspond with reduced cortical representation in the inferior collicular auditory space map, first supported by

Withington and colleages (1994) with guinea pigs. The biological hardware supporting hearing requires activation from the environment in order to develop appropriately.

Experience-dependent plasticity, or adaptive plasticity, refers to the reorganization (or non-normative refinement) of previously established neural networks due to experience. Developmental and adaptive plasticity are distinguished by the achievement of normalcy or the presence of deviation. Changes associated with exposure to teratogens, such as alcohol, provide an example of adaptive plasticity. Teratogens are agents which, following embryonic or fetal contact, produce structural abnormalities in an infant. Gestational exposure to alcohol via maternal consumption can produce a specific behavioural profile, referred to as Fetal Alcohol Syndrome (FAS). This profile is known to include hyperactivity, lower intelligence, and increased social difficulties (Steinhausen & Spohr, 1998; Streissguth et al., 1991). Rodent studies have demonstrated that these behavioural consequences appear to be the result of underlying damage, particularly in the cerebellum, hippocampus, and prefrontal cortex (reviewed by Klintsova et al., 2013). As reported, developmental and adaptive plasticity both produce brain change, but the two categories of plasticity represent unique activity-dependent phenomena in the brain.

Plasticity is age-dependent

The effect of experience on the brain is age-dependent. The different cortical areas grow and organize themselves continuously, sometimes simultaneously and, other times, independent of one another. When growth rates are at their highest for a given region, the area is considered to be undergoing a *critical period* of development. This critical development can refer to various processes of change, including neurogenesis, dendritic arborisation, and synaptogenesis. When the rate of change stabilizes, the critical period is considered complete, and the opportunity for

plastic change is reduced. All regions of the brain follow individual developmental timelines. As such, specific cortex vulnerability to change is dictated by age. Although change remains possible across the lifespan, the increased propensity for change in the developing brain supports the large learning curve seen in early development, in terms of behaviour and memory.

The particular capacity for plastic change demonstrated by the young can have negative consequences during early development, under non-normative circumstances. The ability to communicate is a primary example. Language development is hierarchical, in that the acquisition of certain speech elements is essential for the development of later elements. This is the case with speech perception preceding speech production, as the degree of perception achieved before the age of 7 months is positively correlated with the amount of speech production between the ages of 18-24 months (Tsao et al., 2004). When the opportunity to perceive speech is removed, such as in the case of feral children, they can lose the ability to produce speech (Long, 1990). The exact critical period for communicative ability is unclear due to the rarity of incidences to study this event, however, the capability to acquire language has been pinpointed to late childhood. Some clarification regarding the critical period of language development can be derived from comparing two famous feral children with no exposure to language until they came into contact with researchers. Genie, discovered in the wild at age 12, never gained the production of language, whereas Isabelle, freed from confinement at age 7, flourished under special education and was capable of normal functioning 18 months later (Long, 1990). This discrepancy is explained by cortical region stabilization occurring before the age of 12 yet after the age of 7. Adult language is heavily reliant on the frontal lobes (Schallar et al., 2002), which undergo later development than the majority of the cortex. From this, it can be inferred the age-dependency of language acquisition is determined by the critical development of the frontal

regions. When appropriate stimulation during critical periods is absent or lacking, the decreased potential for plastic change associated with aging results in the loss of normal development.

The higher capacity for plastic change in the young can also be protective during early development. In a rat model of depression, chronic mild stress has been shown to decrease neurogenesis in adults, yet increase neurogenesis in juveniles (Toth, 2008). This protective effect of youth could contribute to the lower prevalence of depressive disorders in children compared to adults (Vasiliadis et al., 2007; Fleming et al., 1989). In considering this differential, the young brain appears to demonstrate greater resilience than the adult brain. Regardless of the extent of youth resiliency or vulnerability in a given situation, there is a need for the consideration of age as a factor in brain change.

Plasticity is location-specific

Given the variance in developmental timelines between the cortical regions, and consequently, the unique propensities for change throughout the brain, activity-dependent plasticity is often seen in particular locations. Additionally, the number of global sensory experiences is limited (e.g. motor training provides specific tactile stimulation). In particular, lasting effects of adaptive plastic change tend to present as focal as opposed to diffuse (Kolb & Gibb, 2011). For example, maternal nicotine use has been associated with a wide range of effects on the newborn including impaired orientation, regulation, and muscle tone difficulties; yet, as the child grows, maternal nicotine use is primarily associated with hyperactivity (Behnke & Smith, 2013). Correspondingly, although gestational nicotine exposure initially disrupts mass nicotinic acetylcholine receptors distributed throughout the nervous system, the widespread nature of effects will localize over-time. Maternal smoking has been shown to impact particular structures, such as the hippocampus and cerebellum, in accordance with the gestational age of

exposure (Dwyer et al., 2008). Due to the variance in regional impact from a given experience, it should be noted the lack of plastic change in a given region does not necessarily mark the absence of functional change.

Plasticity is time-dependent

Permanency is not a required criterion of plastic change. The duration of change is highly reliant on the length of exposure to a given experiential factor. For example, duration and number of incidences of abuse in children has been positively correlated with increased symptomology of depressive disorders and post-traumatic stress disorder (PTSD) (reviewed by Kendall-Tackett et al., 1993). In an attempt to look at the underlying structural differences related to the effect of recurrent stress, the time-dependent stress (TDS) rodent model was modified for exploring PTSD (Harvey et al., 2003). In this paradigm, an intensely stressful event is followed with repeated exposure to situational reminders of the event. Using this model, brain changes following acute stressors have been compared to changes following repeated stressors. The biological evidence supports the behavioural differences between the two scenarios, with findings such as prolonged decreases in dopamine concentrations in the frontal cortex following repeated stress exposure only (Harvey et al., 2006). Chronicity of stimulus exposure must be considered when looking for lasting change.

Understanding how these four factors (experience, age, location and time) modulate activity-dependent plasticity allows for ongoing examination of circumstances involving brain change. Consideration of the principles is important moving forward in terms of experimental design and data interpretation. With these principles acting as guidelines, the behavioural and biological facets of plasticity continue to be identified.

1.3 Current understanding of perinatal HI-induced plasticity

Brain damage can be a consequence of plastic change following reduced oxygen conditions during the perinatal period. Typically, reduced oxygen intake to the blood, or hypoxia, involves reduced blood supply to a given tissue, or ischemia. In a hypoxic-ischemic (HI) event, cellular energy is both limited and poorly circulated. Premature infants demonstrate a particular likelihood of experiencing HI events, due to circumstances surrounding birth and lung underdevelopment (Vogel et al., 2015). Prematurity, according to the World Health Organization (2015), is defined as <37 weeks gestation, with a designation of early prematurity in births with <32 weeks gestation. Prior to recent medical advances, these reduced gestational periods were commonly associated with death following perinatal HI (Volpe, 2009). Now premature infants are experiencing survival rates as high as 85% (Volpe, 2009), consequently incurring more functional consequences. The functional consequences of HI include cerebral palsy, epilepsy and a wide range of developmental and cognitive delays (Hossain, 2005; Lorenz et al., 1998). As such, HI is considered the leading cause of injury in the infant brain (Hossain, 2005). There have been numerous attempts at intervention following perinatal HI, including agents such as barbiturates and glucocorticosteroids to reduce HI-induced cerebral edema, and procedures such as hypothermia, to reduce metabolic needs (Vannucci & Perlman, 1997). To date there is no standardized, empirically supported method of care to modulate HI brain damage in infants.

The initial presentation of HI injury in infancy is the clinical syndrome hypoxic-ischemia encephalopathy (HIE). In order for an infant to meet criteria for HIE, they require a persistent Apgar score of 0-3 for longer than 5 minutes, exhibited neurologic sequelae (eg. seizures), dysfunction of multiple organs and a pH <7 in the umbilical artery blood (Verklan, 2009). Underlying the behavioural changes associated with HIE are a range of anatomical differences,

dependent upon the severity of the injury and the infant's gestational age. These anatomical alterations have been investigated with human imaging studies, as well as animal models. In full term infants, tissue damage is most apparent in the ventrolateral thalami, the posterior limb internal capsule (PLIC), the posterior lateral putamen, and the periolandic cortex, as these regions have the highest energy demands and experience the greatest energy deficit in the absence of oxygen (Izbudak & Grant, 2011). More severe injuries will correspond with damage across the corticospinal tracts, the dorsal brainstem, and the hippocampi (Izbudak & Grant, 2011). In preterm infants, HI injury will present similarly to that following full term gestation, with additional deep grey matter, brain stem and basal ganglia injury (Logitharajah et al., 2009). When HI injury occurs between 23 and 32 weeks gestation, the infant is at increased risk of developing periventricular leukomalacia (PVL) (Back et al., 2001). At best, PVL entails dysregulation of oligodendroglia precursors and, at worst, diffuse necrosis of cerebral white matter (Volpe, 2001). The two most common locations of injury are the posterior periventricular white matter around the upper-outer angles of the lateral ventricles and the frontal white matter adjacent to the foramina of Monro (Izbudak & Grant, 2011). As shown, shorter gestational periods are associated with more diffuse damage. The literature describes late prematurity as a period of both HI susceptibility and regionally specific damage.

The rat model has been used the most extensively in the literature, to explore HI damage (Kolb & Gibb, 2011). Perhaps the greatest asset of this model is the majority of the rat's maturation is completed postnatally, allowing for overt manipulation during critical development. As with all circumstances of change, the four principles of plasticity apply to any paradigm involving the rodent model. Consideration towards the event being modelled influences the manipulation applied to the rat. A common approach to explore perinatal HI is the

Rice-Vannucci rat model, best suited for replication of an acute event such as the umbilical cord wrapping around the infant's neck during delivery. This model is created by ligation of the right common carotid artery of a rat followed by several hours of exposure to reduced oxygen levels (Vannucci & Vannucci, 2005). A less common model of HI involves chronic exposure to reduced oxygen levels, in combination with the incomplete lung development seen in the rat's 1st month of age (Powell & Whitney, 1980). Although supported less in the literature, the chronic hypoxia exposure model best replicates HI events associated with prematurity. When operationally defining "chronic exposure" the exact duration required to produce damage is not explicitly understood. Prolonged duration of hypoxic conditions is both more likely to show damage and more appropriate to capture the extended periods of inadequate oxygen intake associated with HI in preterm infants.

Despite the extensive comparative data between rats and humans relative to other animal models, the exact parallel drawn between the premature infant and the rat pup is a subject of debate. Dobbing and Sands (1979) pinpointed postnatal day (PND) 7 as the rat's age-equivalency to prematurity based on brain weight as a percentage of body weight. From that publication, many researchers used this time point without question, despite its lack of consideration for critical periods. Kolb and Whishaw (1998) determined PND 7 to PND 10 as equivalent to the third trimester based on degree of dendritic branching in all cerebral tissue. Romijn and colleagues (1991) defined PND 12 as equivalent to full term based on overall cerebral cortex development (synapse concentrations, enzyme activity, etc.) and Vannucci and Vannucci (2005) denoted PND 7 as equivalent to the end of early prematurity based on histological similarities (e.g. white matter myelination) with a human fetus. As PND 7-PND 10 in the rat can be considered representative of later gestation prematurity in the infant (see Figure 1), this time

period represents the rat's greatest vulnerability for localized HI-induced damage. Aside from the historical roots of the hippocampus in plasticity research, the hippocampus is a commonly targeted structure in HI study because of its link to HI's functional consequence of cognitive impairment (Christoffel et al., 2011). Additionally, in the rat model, the hippocampus becomes increasingly susceptible to HI-induced damage between the ages of PND 3 and PND 13 (Towfighi et al., 1997). The rat pup exposed to hypoxic conditions between PND 7 and PND 10 best models HI in a late-gestation premature infant, and the hippocampus is a strong candidate for investigating the plastic change in this model.

The rat model has been used to explore cellular changes associated with HI. HI injury occurs in two phases of molecular cascades, each in response to cellular energy failure (see Figure 2). The initial failure is the direct consequence of reduced adenosine triphosphate (ATP). ATP is a storage molecule for cellular energy and its production is made possible through oxygen. Without energy, cellular metabolism falters, including the Na^+/K^+ ATP-dependent pump contributing to the maintenance of resting potential (Malek et al., 2005). Disruption of this pump results in depolarization, which is followed by the release of glutamate into the synaptic cleft. Glutamate has multiple biological purposes, including excitatory neurotransmission in the cerebral cortex, providing cellular energy, and rapid-acting neurotoxicity (Choi et al., 1987). With prolonged loss of oxygen, the cellular environment enters a state of *excitocity*, or neuronal death due to excessive excitatory stimulation, primarily through the activation of glutamate receptors (Johnston et al., 2001). Glutamate in the synaptic cleft activates ionotropic glutamate receptors (divided into N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethul-4-isopropenylpyrrolidine (kainite) receptors) and metabotropic receptors (divided into groups I, II, and III). This activation

triggers an influx of calcium, via gradual release from intracellular calcium stores. Elevated calcium levels trigger downstream neurotoxic cascades, producing a relatively immediate primary wave of cell death. In the rat model of perinatal HI, this first injury occurs within 3 hours of an HI event (Northington et al., 2011). The initial energy failure is responsible for the principle damage associated with HI.

Inflammatory processes heavily influence secondary injury. Although the brain was once considered immune privileged, inflammation is now seen as fundamental to brain injury (Ziebell & Morganti-Kossman, 2010). The immune system acts as an interpreter of external stimuli, modulating brain processes in order to produce the most appropriate response given the circumstances. In perinatal HI, an inflammatory response is induced following a latent energy failure, in response to reoxygenation. This second energy failure is seen 6-24 hours after the HI event. Once the inflammatory response is initiated, T cells, or the primary cells of the immune system, infiltrate the CNS (Lehmann, 1998). Extracellular glutamate in the brain acts on T cells, which also express ionotropic and metabotropic glutamate receptors (Kostanyan et al., 1997). Glutamate activation mediates calcium signalling in T cells and, thereby, chemical messenger production and release. There are multiple types of T cells, secreting different chemical messengers referred to as cytokines. Cytokines are polypeptides allowing for non-direct cell-to-cell contact within the immune system. HI initially favours the activation of $CD4^+$ T helper (Th) 1 cells, seen through an increase in the concentration of Th-1 produced-pro-inflammatory cytokines following an HI event (Bona et al., 1999; Yuan et al., 2010). Pro-inflammatory cytokines are designated as such due to their role in furthering inflammatory processes. The magnitude of secondary injury is driven by the extent to which inflammation spreads.

A homeostatic balance in immune signalling is typically maintained through the up-regulation of anti-inflammatory cytokines in response to high concentrations of pro-inflammatory cytokines. This balanced communication system assists in the establishment of efficient cell networks, an obvious beneficial role of the inflammatory response. It is when balance is not maintained, as in the case of infants born premature, that pervasive, excessive inflammation produces damage. HI events are marked by high levels of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interleukin 6 (IL-6; Aly et al., 2006). HI influences other cytokines, such as the anti-inflammatory cytokine interleukin 10 (IL-10), but to a lesser extent (Vuilleyfroy de Silly et al., 2015). The roles of the various cytokines in the molecular cascade following HI vary, including the induction and suppression of other cytokines and the activation of molecular pathways producing cell death. TNF-α, IL-1β and IL-6 are most commonly linked to HI, as their elevated concentrations are correlated with brain injury severity in hypoxia-exposed infants (Aly et al., 2006). IL-6 is most routinely and strongly elevated in cases of neonatal HI (Aly et al., 2006; Hansen-Pupp et al., 2008; Silveira & Procianoy, 2003). Therefore, although there are many cytokines implicated in HI injury, IL-6 is an important target for investigating HI-induced inflammation.

IL-6 activates two major signalling pathways leading to apoptosis (see Figure 3). The first involves a member of the signal transducer and activator of transcription (STAT) protein family. Various members of the STAT family, once bound by JAK, target gene transcription and, consequently, cell growth (Aaronson & Horvath, 2002). IL-6 induces STAT-3 via the Janus kinase (JAK)-transcription 3 (STAT3) pathway. The second pathway is the SHP2-Gab-Ras-extracellular signal-regulated kinase (Erk)-mitogen-activated protein kinase (MAPK) pathway. MAPK mediates various proteins, via changes in phosphorylation, involved in apoptosis,

including Bcl-2 and caspase 9 (reviewed by McCubrey et al., 2007). The Bcl-2 family of regulator proteins modulate cell death, through mechanisms such as the activation or inhibition of caspase-9, a protease enzyme critical to apoptosis (Li, 1997; Los, 1999). The MAPK pathway is also required for the activation of hypoxia-inducible factor-1 α (HIF-1 α), the most biologically viable transcription factor in low oxygen states. HIF-1 α supervises internal oxygen homeostasis, and communicates disturbances through activation of cell-signalling molecules such as vascular endothelial growth factor (VEGF) (Semenza et al., 1997). Following this activation, growth factors go on to further mediate the STAT-JAK pathway. The second wave of cell death, produced through IL-6's influence on cell growth at the nucleus, can extend over years. It is this secondary brain injury that has been implicated in the most severe consequences of perinatal HI, such as cerebral palsy. Though the mechanisms of cell death able the immune system in eliminating cells altered by stress-associated plasticity, disruption of crucial cell networks is inevitable.

1.4 Summary

Although the demonstrations of plastic change continue to grow in number, there still is uncovered ground. Additionally, research makes continued improvements in understanding how these changes occur. Foundational to plasticity work is an understanding of what has been discovered to date, and the multiple levels (behavioural, anatomical, and microscopic) at which change has been shown. Equally important is consideration of the guiding principles plastic change will follow, specifically activity-dependency, age-dependency, location-specificity, and time-dependency. Finally, in order to effectively contribute to the literature surrounding a particular circumstance linked to plasticity, the recognized mechanisms of change must be reviewed. These mechanisms have particular relevance in circumstances such as perinatal HI,

where means of damage mitigation are unclear. HI injury demonstrates the impact of neuromodulation from the immune system, implicating the relevance of inflammation in potential interventions.

Chapter 2: The gut's inflammatory response and its relation to brain plasticity

Inflammation is a systemic process, divided into two subsets by primary function. The *innate immune response* is chiefly geared towards generic protection and the *adaptive immune response* is particularly reactive to external threat. Inflammation in the body was traditionally conceptualized as irrelevant in the context of brain function, as the CNS was considered incapable of hosting or producing an inflammatory response. This assumption changed when Wekerle and colleagues (1987) showed activated lymphocytes could freely cross the blood brain barrier (BBB). Lymphocytes, which include T cells, are cellular messengers of the adaptive immune response. Lymphocytes are produced in primary lymphoid tissue, such as bone marrow, and circulated throughout the body. T cells are specifically involved in *cell-mediated immunity*, which has a primary function of cytokine release. In light of the BBB permeability in response to approaching inflammation, inflammatory signalling throughout the body is an important consideration in the functioning of the CNS.

There is growing evidence inflammatory signalling in the brain is mediated by the gut, and vice versa. In humans, 70-80% of an individual's lymphocytes are confined to gut-associated lymphoid tissue (Mayer, 2012). These lymphocytes modulate intestinal behaviour via interaction with the enteric nervous system (ENS). The ENS is a network of neurons implanted in the lining of the gastrointestinal system. The purpose of the ENS, or the "second brain," is to direct motility and chemical secretions in the gastrointestinal tract. The ENS and CNS are interconnected structurally, and share a developmental origin and a characteristic inflammatory response to stress. From this relationship, it can be hypothesized that modulation of inflammation in the gut holds implications for inflammatory processes in the brain.

2.1 Brain-gut axis

The overall connection between the brain and the gut is commonly referred to as the "brain-gut axis." Before biological evidence could support the relationship, behavioural evidence implied communication between the two systems. The earliest clinical observations of the gut influencing the brain noted visceral effects, such as diarrhea and abdominal discomfort, were associated with strong emotions like anxiety and fear (Campo et al., 2004). The first scientific theory to address this relationship combined the individual ideas of James and Lange. The James-Lange theory of emotion described physiological changes as essential precedents for emotional shifts. This bottom-up perspective on affective experience depicted emotions as consequences of physical responses communicated to the brain (reviewed by Mayer, 2011). Although the simplicity of the attribution would be debated, the inclusion of physiological response in the production of emotion was expanded upon. Scholars such as Pavlov and Cannon would examine the parallel, top-down relationship of the brain-gut axis and demonstrate emotional reactivity altering the function of the digestive tract (reviewed by Azis & Thompson, 1998). Research continues to support the bi-directionality of influence between the CNS and ENS, with brain lesions producing changes in gut function (Wood et al., 1985) and alterations in physiological response correlated with variance in subjective affect (Mauss et al., 2005). Although the means of influence were initially unclear, a symbiotic relationship between brain and gut has been documented widely in the literature.

The developmental origin of the brain-gut connection lies in the cellular differentiation of the neural tube. During embryonic development, the neuroepithelium differentiates from the neural tube to produce the CNS, whereas the neural crest differentiates from the neural tube to form the ENS (Yntema & Hammond, 1954). The ENS precursor cells follow a rostro-caudal

progression down the vagus nerve, one of 12 cranial nerves and the longest nerve in the autonomic nervous system (ANS; Young et al., 1998). As the neural crest cells move downwards, the gastrointestinal tract undergoes development, beginning with the stomach at approximately 4 weeks gestation in humans (Deren, 1971). The vagus nerve moves to innervate the bowel during fetal development, in order to coordinate reflexes such as sucking and swallowing immediately at birth (Porges & Furman, 2011). In the rat, the vagul axons have been shown to enter the stomach by embryonic day 12 (Rinaman & Levitt, 1993), establishing the route of ENS development. As the neural crest cells differentiate into neurons and glial cells in the gut, the layers of epithelial cells in the tract are progressively replaced (Ménard & Arsenault, 1990), thereby embedding the developing ENS within the layers of gut tissue. When development is complete, the ENS will consist of 200 to 600 million neurons, outnumbering the spinal cord (reviewed by Mayer, 2012). With both efferent and afferent neurons, the ENS is designed to act both independently of, and interdependently with, the CNS.

Interdependence between the brain and the gut is maintained with bi-directional communication throughout the lifespan. As means of connection, the CNS and ENS use both branches of the ANS, the sympathetic nervous system (SNS) and the parasympathetic nervous system (PNS). In the gastrointestinal system, the SNS is primarily involved with inhibitory behaviours including slowing motility and secretion, whereas the PNS has a larger role in immunity, such as the activation of macrophages, white blood cells which release cytokines (Pavlov & Tracey, 2005). The vagus nerve, which first establishes the brain-gut axis, remains the primary source of interconnection between the CNS and the ENS. The vagus nerve is a component of the PNS and it innervates almost the entire gut except the distal portion of the colon. CNS gut innervation is primarily sourced by the hypothalamus and the amygdala. In turn,

these structures are relayed messages from the gastrointestinal system through the nucleus tractus solitaries in the medulla of the brain stem. Inflammatory signalling at either end of the feedback loop can influence the line of communication between the CNS-ENS. Due to this maintained contact, influencing the inflammatory state of the gut can impact the inflammatory state of the brain.

2.2 Inflammation in the preterm gut

Inflammation is an expected component of typical development in the gut, specifically development of the microbiome (Dominguez-Bello et al., 2011). The microbiome, or the specific distribution of microorganisms within the gut, is a defensive shield against external stimuli. The microbiome is primarily developed through exposure to the mother's flora (Marcobal & Sonnenburg, 2012), including contact with bacterial species in the birth canal during vaginal birth and when breast-fed. An inflammatory response is expected from exposure, as the various species colonize the gut. The release of proinflammatory cytokines increases the permeability of the intestines (Drewe et al., 2001), contributing to greater bacterial influx and, consequently, more excessive gastrointestinal inflammation. The term infant implements preventative tactics against persistent inflammation via the intestinal epithelium. This prevention includes a reduction of enzyme complexes required for the activation of the nuclear factor kappa-light chain-enhancer of activated B cells (NFκB) pathway (Claud et al., 2004). The NFκB pathway produces TNF-α, IL-1β and IL-6, and is considered a first responder to aversive stimuli. This prevention is made possible by appropriately developed defense mechanisms.

This pre-emptive reduction of transcription factors is not seen in preterm infants. As prematurity is often associated with caesarean delivery and formula feeding, the preterm microbiome has vital missing organisms (Goldman et al., 1978) and lower microbiome diversity

overall (Schwiertz et al., 2003). This weakened defensive shield is then exposed to atypical microorganisms in the hospital environment. The bacterial influx over-stimulates the NFκB pathway through Toll-like receptors (TLRs). TLRs are recognition molecules that respond to a breadth of stimuli, with TLR4 being the most widely understood (Doyle & O'Neill, 2006). Left unmitigated, the excessive inflammation can produce cell death using the same molecular mechanisms seen in perinatal HI. The microbiome then becomes critical to certain circumstances of pervasive inflammation in preterm infants.

An immature microbiome is cited as a potential cause of necrotizing enterocolitis (NEC) in circumstances of prematurity (Elgin et al., 2016). NEC preferentially targets preterm infants and is considered the most commonly acquired gastrointestinal tract disease in this population (AlFalah & Anabrees, 2014; Ganguli et al., 2013). The range of clinical presentations for the initial stages of NEC include feeding intolerance, bloody stools, abdominal distention/soreness, temperature instability and excessive fatigue (reviewed by Thompson & Bizzarro, 2008). Unchecked inflammation produces necrosis of the gut and bowel. For infants afflicted with NEC, there is a 20-40% mortality rate outside of morbidities ranging from intestinal failure to neurodevelopmental dysfunction (Kosloske, 1994; Hintz et al., 2005). These disabilities share commonality with HI injury, as 20% of preterm NEC patients develop cerebral palsy and 36% some form of cognitive impairment (Rees et al., 2007). These shared consequences are indicative of common tissue damage following both HI and the development of NEC. This is supported by cases of periventricular white matter damage in infants diagnosed with NEC (Bedrick, 2004). Given the communal tissue damage and functional outcomes, it can be inferred that similar mechanisms of injury are activated, likely through inflammation, in both clinical circumstances. Manipulating these mechanisms through a more sophisticated microbiome could reduce the

gastrointestinal consequences seen in NEC and potentially modulate inflammatory consequences in the brain.

A healthy infant microbiome does not have a precise bacterial gut composition and the species present vary between individuals to some extent. There is some understanding, however, regarding organisms critical to gut health. The first exogenous colonizers are typically streptococci, staphylococci, and enterobacteria (Rotimi & Duerden, 1981; Yoshioto et al., 1991). The species exert different immune effects through TLR4. TLR4 responds to the presence of bacteria, first demonstrated by Sen & Baltimore (1986) with the gram-negative bacterial species component lipopolysaccharide (LPS). Gram-negative bacteria, such as enterobacteria, are typically conceptualized as pro-inflammatory, whereas Gram-positive bacteria, such as staphylococci, are often considered anti-inflammatory. Gram designation is based on the structure of the bacteria's cell wall. The two types are identified with crystal violet dye staining, with gram-positive bacteria identified through retention of the dye and gram-negative bacteria by the lack of stain. Given what is known regarding missing strains in the premature gut, it is reasonable to assert bacterial supplementation of Gram-positive bacteria could potentially modulate inflammation in premature infants.

2.3 Probiotics

The incidence of NEC is drastically reduced in breast-fed preterm infants relative to those who received formula (McGuire & Anthony, 2003). Breast-fed infants are distinguished from formula-fed infants by higher intestinal concentrations of probiotics. Probiotics are defined as live, gram-positive, non-pathogenic bacteria that colonize the gastrointestinal tract and provide benefit to the host. When first investigated for use in NEC treatment, there were several hypotheses regarding their potential benefit, including fortification of the intestinal barrier

against bacterial invasion (Matter et al., 2001), competitive interaction with pathogens (Reid, 2001), and initiation of anti-inflammatory processes (Link-Amster, 1994). Although likely from a combination of all three benefits, probiotic supplementation of the microbiome has proven successful in the reduction of gut inflammation.

Bacterial supplementation is best supported by the lower incidence rates of NEC in preterm infants administered probiotics (Lin et al., 2008; AlFalah & Anabrees, 2014). This supplementation can provide benefit in addition to breast-feeding. As a result of these findings, probiotics have been routinely administered to preterm infants across the world (Janvier et al., 2014; Ofek et al., 2014). Probiotics are considered generally safe and well tolerated (Carey & Boullata, 2010), with no demonstrated negative effects on developmental measures such as weight gain (Mihatsch et al., 2012; Underwood et al., 2015). The first long-term assessment of functional outcomes did not find a relationship between probiotic administration and harmful outcomes, such as rates of disability, at a 3-year follow-up (Chou et al., 2010). The authors did not find improved neurodevelopmental outcomes for premature infants who received probiotics relative to those that did not but they only assessed visual and auditory impairment. To date, there has been no comprehensive assessment of the enduring effect of probiotics on gut inflammation-induced neurological consequences such as cognitive impairment and cerebral palsy. Probiotics appear to be beneficial for gut inflammation, albeit the complete effects of probiotics are incompletely understood.

The two most empirically supported genera of bacteria in the treatment of intestinal inflammation in preterm infants are lactic acid bacteria (LAB), such as *Lactobacilus acidophilus/L. acidophilus,* and bifidobacteria, such as *Bifidobacterium infantis*/*B. infantis*. Administration of both probiotics to immature intestinal tissue has been shown to reduce

elevated cytokine levels, specifically IL-6 (Ganguli et al. 2013). Both are endogenous bacteria, present in the early stages of intestinal colonization (Link-Amster et al., 1994; Arciero et al., 2010). As other bacteria move into the gut, bifidobacteria must be prevalent for normative bacterial composition development (Boesten et al., 2011). Prematurity, however, is associated with a marked absence of bifidobacteria, lasting months post-delivery (Westerbeek et al., 2006). Supplementation of this bacterial species seems to partially restore symbiosis, reducing inflammation. *B. infantis,* specifically, demonstrates superior anti-inflammatory abilities relative to other organisms, such as *L. acidophilus* (Ganguli et al. 2013; Ait-Belgnaoui et al., 2012) and has been shown to colonize the intestines of premature infants better than the other members of its family, such as *B. lactis* (Underwood et al., 2015). Clinical trials have heavily relied on *B. infantis* when exploring the mitigation of NEC-induced inflammation.

Although understanding of neurodevelopmental outcomes following probiotic administration is lacking in the context of NEC, it is absent from the literature regarding inflammatory conditions outside of the gut. This paucity of research exists, despite a growing interest in the influence of microbial gut organisms on CNS function. Experimental manipulation of gut microbial environments in rats has produced changes in stress behaviours (Crumeyrolle-Arias et al., 2014) and cognitive function (Davari et al., 2013). Probiotic administration has also been shown to impact immunity in the brain, including TNF-α, IL-1β and IL-6 mRNA expression in the hypothalamus (Ait-Belgnaoui et al., 2012), an apparently vagally mediated effect (Bercik et al., 2011). Although evidence supports the use of probiotics as an intervention for conditions producing inflammation in the brain, such as perinatal HI, the efficacy of probiotics in mediating HI-induced CNS inflammation remains to be seen.

2.4 Summary

The gut and the brain respond to adverse circumstances with the same inflammatory processes and the same mechanisms of plasticity, sometimes resulting in cell death. Although CNS inflammation is difficult to mitigate, the gut provides a direct line of access to the brain because of their reciprocal influence. Reducing inflammation in the gut is a viable target for reducing inflammation in the brain. In premature infants, probiotics have been shown to be effective in the reduction of gastrointestinal inflammation, thereby implicating probiotics as potentially useful for decreasing CNS inflammation in the same population.

Chapter 3: An exploration of the effect of *B. infantis* on hippocampal IL-6 levels

3.1 Introduction

The plasticity associated with injury, such as that seen in circumstances of perinatal hypoxia ischemia (HI), has been shown to alter the developmental trajectory of the brain, prompting detrimental functional outcomes. Structural changes are seen in brain regions such as the basal ganglia, the brain stem, and the hippocampus, following HI events in infancy (Logitharajah et al., 2009). Functionally, these changes translate into varying degrees of motor dysfunction, including cerebral palsy, (Forslund, 1992) and cognitive impairment (McCormick et al., 1992). Infants born premature, or with a gestational period of <37 weeks (WHO, 2015), have an increased likelihood of experiencing HI events relative to infants born full term (Vogel et al., 2015), with lung underdevelopment being a primary factor in the increased incidence of HI. Premature infants also demonstrate poorer functional outcomes following HI events, relative to full term infants, given the higher rates of development their brains are undergoing. Currently, there are no empirically based interventions for HI injury. Given the dispropotionate prevalence of HI, and adverse outcomes of HI, associated with premature infants, the need for an intervention with this population is particularly pertinent.

Critical to the development of an intervention for HI, in conjunction with prematurity, is an understanding of the inflammatory response evoked in a preterm infant following HI events. Inflammatory processes, induced by cellular energy failure in the absence of oxygen homeostasis, dictate the severity of HI injury. Left uncontrolled by the immature immune system of the premature infant, the HI-induced inflammatory response results in excessive levels of pro-inflammatory cytokines. In particular, HI favours the release of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (Aly et al., 2006). Additional cytokines, both pro-inflammatory and anti-

inflammatory, are involved with the cellular repercussions of HI, albeit less consistently (Vuilleyfroy de Silly et al., 2015). Of all the cytokines associated with HI, elevations of IL-6 concentrations are most commonly cited in the literature, due to routine and strong elevations following HI events (Aly et al., 2006; Hansen-Pupp et al., 2008; Silveira & Procianoy, 2003). This correlation situates the reduction of IL-6 as an appropriate target for mitigation of HI-induced inflammation.

The inflammatory state of the brain is highly influenced by the inflammatory state of the gut, via structural and communicative interconnection (Mayer, 2012) between the two systems. In preterm infants, both CNS inflammation and ENS inflammation are marked by similar elevations of pro-inflammatory cytokines, including IL-6 (AlFalah & Anabrees, 2014). In circumstances of gastrointestinal inflammation, such as NEC, probiotics have proven effective in reducing IL-6 levels in premature infants (Ganguli et al. 2013). In particular, *Bifidobacterium infantis /B. infantis* had demonstrated superior colonization of the infant gut (Underwood et al., 2015) and the strongest mediation of the immune system relative to other bacterial species (Ait-Belgnaoui et al., 2012; Ganguli et al. 2013). Given this, it can be proposed that probiotic administration may result in the reduction of inflammation in the brain through impacting the inflammatory state of the gut. Of the various probiotics, *B. infantis* presents as a strong candidate for intervening with HI-induced, CNS inflammation in the premature infant.

The objective of this study was to examine whether IL-6 levels in the brain would be impacted by probiotic administration, and we chose to explore inflammation in the hippocampus given its relevance to HI injury. We opted for the use of a chronic oxygen deprivation model of HI, despite its lower prevalence in HI literature relative to the acute hypoxia exposure model associated with the Rice-Vannucci method. While the chronic hypoxia exposure model is less

well characterized in the literature in terms of neurodevelopmental consequences, in comparison to models using acute exposure, it can be conceptualized as a more appropriate model for the most common circumstances surrounding HI events with premature infants (ie. immature lungs and chronic reduced oxygen intake). We hypothesized the neurodevelopment of pups reared in hypoxia would be negatively impacted by that environment. Neurodevelopment was measured through brain weight to body weight ratios, brain size and body growth over time. Given the literature pertaining to the negative effects of reduced oxygen on development, we expected those reared in hypoxic conditions to exhibit higher brain to body weight ratios, smaller brains, and lower rates of growth, relative to animals in normoxic conditions, thus supporting our chronic exposure model of HI. We hypothesized that IL-6 levels would be elevated in the hippocampi of animals reared in hypoxia, relative to those in normoxia, because of the association between elevations of IL-6 and HI events and the correlation of HI injury and plastic change in the hippocampus. We hypothesized animals in hypoxia would have reduced IL-6 concentrations, relative to controls, following probiotic administration, due to an anti-inflammatory effect of *B. infantis.* We expected double control animals (normoxia, no probiotics) to have the lowest levels of IL-6 and hypoxic animals that did not receive the intervention to have the highest levels of IL-6, given the literature's association with IL-6 elevations and inflammatory states and the expected effect of probiotic administration.

3.2 Materials

Animals

In total, a sample size of 72 pups was used for this experiment. To produce litters, adult, female Long Evans rats were paired with adult, male Long-Evans rats from the Ivanco Breeding Colony. The pairs were housed individually in a colony room at the University of Manitoba in

polypropylene cages (8 x 18 x 10 inches) under a controlled 12-hour light-dark cycle. The temperature of the colony was approximately 22°C and the oxygen level was approximately 21%. Water and food were available *ad libitum*. Females were weighed every day, as weight gain due to pregnancy is expected by the 10th day of the 21-day gestation period. Once pregnancy was established, males were removed and females were housed individually during the remainder of the gestation period.

At birth, pups were divided between four groups: 1) animals in normoxia who received probiotics, 2) animals in normoxia who did not receive probiotics, 3) animals in hypoxia who received probiotics, and 4) animals in hypoxia who did not receive probiotics. In the end, group 1 has 18 pups, group 2 had 18 pups, groups 3 had 12 pups, and group 4 had 24 pups; the sex distributions can be found in Table 1. After the first 12 pups from the hypoxic, probiotic treatment group were sacrificed, dissection determined apparent gastrointestinal damage in 3 animals. The damage was consistent with the appearance of NEC and/or sepsis (see Figure 4). Concerned about a negative interaction between reduced oxygen levels and *B. infantis,* no more animals were exposed to both the probiotic and the hypoxic chamber. This, and the fact that litter sizes varied, accounts for the unequal group sizes.

Groups 1 and 3 were run after all the tissue from Groups 2 and 4 had been collected, as clinical experience had demonstrated probiotics have the ability to colonize not only those administered the treatment, but also those in the surrounding vicinity. The day each dam gave birth was denoted as PND 0 for that litter. Pups were not separated from mothers, outside of daily weighing, and handling was kept to a minimum for the duration of the experiment in order to reduce stress. All procedures were within the standards set by the Canadian Council on

Animal Care, and were approved by the Protocol Management and Review Committee at the University of Manitoba (F#15-015).

Hypoxic exposure

On PND 3, animals within Groups 3 and 4 were placed in a hypoxic environment, whereas Groups 1 and 2 remained in normal oxygen levels. Groups remained in the given environmental condition until PND 10. Hypoxia was produced placing cages containing dams and litters (no more than two cages at a time) into an acrylic and wood chamber (20 x 24 x 24 inches) with a monitored 10% +/- 1% oxygen delivery. This oxygen level had been determined from previous work conducted in the lab. When the chamber was opened daily, oxygen levels would return to normal. A hypoxic state was re-established over a period of several hours, as oxygen levels required time to drop and settle at ≈ 10%. Additionally, when pups were removed from the chamber for daily weighing and solution administration, they were exposed to normal oxygen concentrations for approximately 40 minutes, as both cages were handled before resealing the chamber.

Probiotic administration

During daily weighing from PND 3 through PND 9, pups were administered either the probiotic intervention or the distilled water control, depending on group assignment. The probiotic intervention consisted of *B. infantis* organisms in combination with distilled water. Administration occurred via squirting the appropriate solution into the mouth of the pup with a feeding needle. As a therapeutic dose of probiotics has not been previously defined for an animal model of perinatal HI, each pup received a dose comparable to that given to premature infants as an intervention, as per Underwood (2014). This dose was approximately 5×10^6 colony-forming units/g body weight/day.

3.3 Methods

Tissue Preparation

On PND 10, all pups were weighed and sacrificed via decapitation. Brains were removed and weighed. Blood was collected via syringe from the body of each pup to measure corticosterone levels, a measure of systemic inflammation, and to ensure tissue analysis captured the inflammatory state of the brain as opposed to inflammation in the body. Brains were swiftly removed and stored at -80°C until analysis. One hemisphere of the brain was used for this study's analysis and one was fixed. In the analysis hemisphere, the hippocampus was removed from the remainder of the cortex.

Tissue Analysis

In order to quantify the volume of sample required to determine protein concentration, a Bradford assay was run. Protein samples were lysed in order to degrade cell membranes, then ground and centrifuged. In accordance with the protocol, samples were combined with Bradford reagent and read using an iMark™ microplate reader (Bio-Rad). Through plate readings, the volume of sample required to obtain 5µg of protein was determined in order to standardize the volume of protein for IL-6 quantification.

Enzyme-linked immunosorbent assay (ELISA) was used to assess the concentration of IL-6 in the protein samples (RayBio® Rat IL-6 ELISA kit for lysates). The steps undertaken were defined by the manufacture protocol. Standards were prepared using a dilution series of Standard Rat IL-6 Protein. Both standards and samples were loaded via pipette into an 96-well microplate, precoated with anti-Rat IL-6, followed by incubation at approximately 22°C for 2.5 hours. The plate was then washed four times with Wash Buffer, before adding a detection antibody concentrate to each well. After another four washings, the plate had a 1-hour incubation

period before a Streptavidin solution was added. The plate was washed again, four times, before TMB One-Step Substrate Reagent was added, turning the wells various shades of blue. The blue colour indicated the presence of horseradish peroxidase (HRP) activity, an enzyme necessary in the increased detectability of IL-6. After a 30-minute incubation period in the dark, Stop Solution was added to each well, yielding a yellow colour. The absorbance of the yellow colour was representative of the IL-6 concentration in the given well. The absorbance was read using an iMark™ microplate reader (Bio-Rad) at 450nm. The assay was done in duplicate. For each assay, a standard curve was plotted from the absorbance of the standards.

Blood samples were centrifuged to collect serum. A Corticosterone (Rodent) ELISA kit was used to assess the concentration of serum corticosterone. Pre-made standards and serum samples were pipetted into a microtiter plate precoated with Corticosterone specific antibody. Corticosterone Enzyme Conjugate Solution was added to each well, and the plate was incubated for 2 hours at 37°C. The plate was then washed five times with Wash Buffer, before adding TMB colour reagent to each well, followed by a 20-minute incubation period. Stop Solution was added to each well, which turned each sample and standard yellow. The concentration of the yellow colour was representative of the corticosterone concentration in the given well. The absorbance was read using an iMark™ microplate reader (Bio-Rad) at 450nm. A standard curve was plotted from the absorbance of the standards.

All statistical analyses were completed using Statistica (StatSoft, Tulsa, OK). The brain weight to body weight ratios of the pups were assessed using a general linear 4 x 2 factorial model. The independent variables were group (normoxia with probiotics, normoxia without probiotics, hypoxia with probiotics and hypoxia without probiotics) and sex (male, female) and the dependent variable was percentage. Brain weight was assessed using a 4 x 2 ANOVA model.

The independent variables were group (normoxia with probiotics, normoxia without probiotics, hypoxia with probiotics and hypoxia without probiotics) and sex (male, female) and the dependent variable was weight in grams. Body growth over time was assessed with a repeated measure ANOVA. The independent variables were group and sex, the dependent variable was body weight in g, and the repeated measure was days. The concentration of IL-6 in the hippocampus was assessed with a general linear repeated measures model. The independent variables were group and sex, the dependent variable was IL-6 concentration in μg/mL, and the repeated measure was plate. The measure of plate was produced through running two separate ELISAs on the same samples. The concentration of corticosterone in the blood was assessed with a general linear 4 x 2 factorial model. The independent variables were group and sex and the dependent variable was corticosterone concentration in μg/mL. Post hoc comparisons were done with the Tukey HSD for unequal N test. The α level of significance used was p=0.05.

3.4 Results

During data collection, there were two separate incidences involving the potential for mislabelled tissue. Of the 72 animals, 21 were originally mislabelled. This occurred through the use of inaccurate numbering start points. The results represent the data after an attempt at correctly identifying all tissue samples, however, the results must be considered tentative in light of the labelling uncertainty.

Neurodevelopment measures

For brain weight to body weight ratio, there was a significant main effect of group ($F(3)=88.48$, $p=<0.001$, $\eta_p^2=0.81$). Post hoc comparisons indicated the mean brain weight to body weight ratio for animals reared in normoxic conditions, both with and without receiving probiotics, was significantly lower than the brain weight to body weight ratios of animals reared

in hypoxic conditions, both with and without probiotics. These results indicate a main effect of environmental condition on brain weight to body weight ratio (see Figure 5). There was no significant main effect of sex ($F(1)=1.52$, $p=0.222$), or an interaction between group and sex ($F(3,1)=0.30$, $p=0.827$). For brain weight, there was no significant effect of group $F(1)=1.02$, $p=0.115$), no significant effect of sex ($F(3)=2.06$, $p=0.171$), or an interaction between group and sex ($F(3,1)=2.10$, $p=0.165$; see Figure 6). For body growth over time, there was a significant main effect of group ($F(3)=192.9$, $p=<0.001$, $\eta_p^2=0.88$) and a significant effect of day ($F(6)=541.3$, $p=<0.001$, $\eta_p^2=0.81$; see Figure 7). Post hoc comparisons indicated body weight increased significantly each day, in comparison with PND 3, for both groups reared in normoxic conditions. In contrast, the body weight of animals exposed to hypoxia and probiotics was only significantly different from body weight on PND 3 at PND 9, and the body weight of animals exposed to hypoxia without probiotics was only significantly different from body weight on PND 3 at PND 8 and PND 9.

IL-6

There was a significant main effect of group ($F(3)=6.4$, $p=0.001$, $\eta_p^2=0.23$). Post hoc comparisons indicated IL-6 levels were significantly higher for animals reared in normoxic conditions without receiving probiotics in comparison to animals exposed to hypoxia, both with and without probiotics. There was no significant difference in IL-6 concentration between the two groups exposed to normoxia, or the two groups exposed to hypoxia. There was no significant difference in IL-6 concentrations between the two groups exposed to probiotics. These results indicate an interaction effect between environmental condition and probiotic administration on hippocampal IL-6 (see Figure 8). There was no effect of sex ($F(1)=1.7$, $p=0.197$), or an interaction between group and sex ($F(3,1)=1.5$, $p=0.224$). In addition, there was

an effect of plate ($F(1)=208.9$, $p=<0.001$, $\eta_p^2=0.77$), with IL-6 concentrations higher in plate 2 than plate 1 (see Figure 9).

Corticosterone

There was no significant main effect of group ($F(3)=1.95$, $p=0.125$; see Figure 10), significant main effect of sex ($F(1)=.160$, $p=0.690$), or an interaction between group and sex ($F(3,1)=.864$, $p=0.464$),

3.5 Discussion

Consistent with our hypothesis, animals reared in the hypoxic chamber demonstrated altered neurodevelopment. Hypoxia exposed animals had larger brain weight to body weight ratios. With no significant differences in brain weight at the time of tissue collection, the difference in brain weight to body weight ratios was driven by animals in hypoxia having significantly lower body weights on PND 10. Animals exposed to hypoxia also demonstrated slower growth patterns relative to animals reared in normoxia, only showing significant gains in weight near the end of the 7-day interval as opposed to across the 7-day interval. Contrary to our hypotheses, IL-6 levels were not elevated in the hippocampi of our hypoxia groups relative to our normoxia groups, and *B. infantis* had no effect on IL-6 levels for animals in either environmental condition. There was a significant difference in IL-6 concentrations between plates, with the second plate run indicative of improved technique. Although there were concentration differences between plates, the pattern across groups was consistent between plates. IL-6 levels were highest in double-control animals (normoxia, no probiotics) and lowest in animals reared in the hypoxic chamber, regardless of probiotic administration. Systemic corticosterone appeared unaffected by environmental condition or probiotic administration. Despite the apparent absence of an effect from *B. infantis* on IL-6 levels, there was qualitative

evidence of an interaction between hypoxia and probiotic administration on gastrointestinal health. The guts of 3 pups exposed to hypoxia and probiotics demonstrated indications of sepsis and/or NEC symptomology, an observation made only within this group. Poor gut health was seen in 3 of the 6 animals exposed to hypoxia and administered probiotics.

There are many models that have been explored to replicate the human condition of HI, each possessing its unique advantages and disadvantages. An advantage of the acute hypoxia exposure model is its abundance in the literature, and therefore its high reliability in terms of evidencing brain injury. It has also been associated with altered neurodevelopment, including cerebral weight (Alexander et al., 2014) and the loss of progenitor cells (McQuillen et al., 2003). The exact severity and location of injury, however, has been shown to vary greatly when using this model, highly influenced by developmental age and length of exposure (Northington, 2006), impacting replication. Additionally, acute hypoxia exposure does not appropriately model the primary causal factor of HI in premature infants (lung underdevelopment) and the surgery associated with the Rice-Vannucci method has the potential to produce confounding inflammatory effects. Indication of altered neurodevelopment supports the use of a chronic hypoxia exposure model, which better fits the human condition and has the potential to improve the variability in reported injury using the same model. Although brain weight was unaffected, weight is only one measure of brain development. Delayed growth and differences in weight are indicative of detrimental developmental consequences from our chronic hypoxia exposure. Our results should encourage future investigators to explore HI using this model.

Our results also speak to the complexity of IL-6's role following an HI event. IL-6 is traditionally conceptualized as a pro-inflammatory cytokine, as the cytokine is routinely elevated during inflammatory states. This association, however, is dependent on the abundance of the

protein, interactions with other signalling molecules, and the surrounding receptor compatibility. Under certain circumstances, IL-6 will demonstrate anti-inflammatory properties. A primary determinant in the inflammatory nature of IL-6 is its route of activation. The most common type of activation is referred to as *trans-signalling.* Here, IL-6 produces a protein complex with soluble IL-6 receptors (sIL-6R), consequently attracting glycoprotein 130 (gp130). Gp130 receptors vastly outnumber IL-6 receptors in the body, therefore the addition of gp130 allows for widespread activation (Scheller & Rose-John, 2006). In the absence of gp130, IL-6 fails to communicate with the majority of cells. When the protein complex binds, the subsequent activation is commonly associated with the generation of inflammatory processes (reviewed by Scheller et al., 2011). Therefore, *trans-signalling* is pro-inflammatory. When IL-6 does not involve gp130 for the purpose of activation, this is referred to as *classic signalling*. With this form of activation, IL-6 binds directly to membrane-bound non-signalling α-receptors IL-6R (mbIL-6R). This form of activation has been linked to the induction of anti-inflammatory, or regenerative, processes in organs such as the liver (Rose-John, 2012). Elevations of IL-6 can then be associated with the induction or reduction of an inflammatory response, dependent on route of activation. Given the literature surrounding the association between inflammation and HI and the indications of poorer developmental outcomes, it can be inferred the brains of animals reared in hypoxia were in an inflammatory state. The results then support hypoxia favouring *classic signalling,* or Il-6 as anti-inflammatory, at PND 10, following 7 days of HI injury. Consequently, animals in the hypoxia condition had reduced concentrations of IL-6 relative to the homeostatic levels of IL-6 seen in the normoxia groups and double-control animals were capable of presenting with the highest IL-6 concentrations.

The trajectory of a cytokine's role in an inflammatory response provides additional complexity to understanding cytokine behaviour. Across situations, cytokine levels have been shown to fluctuate over time. For example, in response to a chronic viral infection similar to human immunodeficiency virus (HIV), IL-6 has been shown to peak 1 day post-infection and significantly drop for 24 days before peaking for a second time (Harker et al, 2011). Evidence such as this portrays IL-6 as heavily involved with the induction of inflammation, as opposed to having a primary role in maintenance, suggesting its highest concentrations will be present at the start of an injury course. When looking at a single protein's expression at a given time point, there is a risk of missing the protein's concentration peaks. In order to better understand the involvement of a cytokine following an event, concentration levels need be assessed across time.

Given the labeling issues during data collection, the present results require replication in an independent study prior to major conclusions being made to the scientific community. In future, labelling concerns could be avoided by the primary researcher demonstrating greater attention to detail and through the creation of one combined table, where data would be entered following each tissue collection, as opposed to having separate, individuals data tables for each tissue collection. Although the labelling issues produce uncertainty in the results, the potential significance of these results in future examinations of the role of IL-6 in HI injury suggests there is a need for replication of this study.

Our model of HI appeared to promote negative neurodevelopmental consequences, suggesting chronic hypoxia exposure from PND3-PND 10 produces a valid model of perinatal HI injury in circumstances of prematurity. IL-6 levels were not elevated in the hippocampus following hypoxia, suggesting potential for either an alteration in inflammatory role under the given circumstances or a fluctuation in IL-6 concentration over time. *B. infantis* did not

demonstrate an ability to influence HI-dictated IL-6 concentrations, with the reason being unclear. Our study supports the use of chronic HI exposure in modelling perinatal HI and suggests the role of IL-6 following an HI event is incompletely understood.

Chapter 4: Future Directions

If provided the opportunity, there are directions we would follow in the future. It is possible a mitigating effect of *B. infantis* on CNS inflammation was non-detectable due to the cytokine measured and/or the brain region investigated. Our study was limited by the inability to look at more than one protein, in one cortical area. Moving forward, we would look at different cytokines, in different regions of the brain. Additionally, as the qualitative effects of probiotic administration, in combination with hypoxic exposure, called into question the safety of a probiotic intervention, we would explore the use of probiotics with a weakened immune system in greater detail.

As we looked at one cytokine only, the potential for missing an effect was large. Even with the capacity to look at multiple cytokines, the numerous relevant signalling messengers in an inflammatory cascade make intervening difficult. At any given time following exposure to inflammatory stimuli, one cytokine may hold more relevance than another. It is possible that, although IL-6 has been associated with the early stages of HI injury, it is not the most pivotal cytokine in the inflammatory response following 7 days of chronic hypoxia exposure. There is evidence the production of HIF-1α shifts tissue from favouring the activation of Th1 cells to inducing Th2 activation, which includes the expression of anti-inflammtory cytokines IL-4 and IL-10 (Romagnani, 1991). Furthermore, the anti-inflammatory behaviour of *B. infantis* has been previously linked to the induction of IL-10 (Tanabe et al., 2008). It is possible an effect of *B. infantis* would have been seen in hippocampal IL-10 levels.

It is equally possible an effect on IL-6 concentrations would have been demonstrated in other areas of the brain. The hippocampus, as the figurative seat of learning and memory, was chosen as the site of examination in this study given its functional relevance to the cognitive

impairment associated with perinatal HI. The regions that initially receive input from the gut, however, are the amygdala and hypothalamus. To see secondary effects of probiotic mediation on inflammation in the gut, it may have been more effective to look at regions of the brain more implicated in the brain gut-axis. Moving forward, future investigators interested in the mitigation of CNS inflammation via probiotics should look outside of the hippocampus.

The qualitative observations made on the gut health of animals administered probiotics in hypoxic conditions were concerning. Both NEC and sepsis, a condition arising from excessive inflammation into the bloodstream, involve an unmitigated inflammatory response. With observations suggesting the presence of either NEC or sepsis, a probiotic-mediated reduction of inflammation in this group seems unlikely. Probiotics appeared to worsen the inflammatory state associated with HI. Though probiotics are, overall, designated safe to use, there have been reports of specific risks in immunologically vulnerable populations. Bacteria at even low virulence can cause disease in compromised hosts. Honeycutt and colleagues (2007) terminated their assessment of probiotic efficacy in reducing infection in a paediatric intensive care unit early when 9 of their 31 subjects receiving probiotics developed infections. They were using the Gram-positive bacterial strain *Lactobacillus rhamnosus*. The *Lactobacillus* family has been problematic in other studies as well. Using molecular DNA fingerprinting analysis, the strain has been directly linked to infection, albeit rarely (Land et al., 2005). Although understated, risks associated with probiotic use are evident in the literature.

The risk of probiotic use culminating in sepsis appears to be greater in cases of early prematurity and/or very-low birth weight, potentially due to the immune-compromised infant's difficulty managing the probiotic colonization of the gut (Guenther et al., 2010). To date, no bifidobacterial probiotic strain has been shown to induce sepsis in humans (Whelan & Myers,

2010), yet assessing the effect of probiotics in preterm infants is confounded by small sample sizes and poor health for both treatment and control groups. Additionally, concern over probiotic product quality has been substantiated. Analysis of bacterial composition has found many commercial probiotic products contain unexpected additional lactobacilli and bifidobacteria strains while missing other strains explicitly advertised (Marcobal et al., 2008). Manufacturing commercial *Bifidobacterium* in particular has been shown to be problematic, with concentrations of the genus consistently lower than labelled and often present in the form of dead cells (Aureli et al., 2010). Most relevant to this study is the possibility of bacteria dying during administration, due to its need for refrigeration. Ultimately, our results echo the speculation of others surrounding reconsideration of the classification of probiotics as drugs under physician control.

Moving forward, the results of our study suggest looking outside of hippocampal IL-6 levels for a CNS effect of probiotics. In particular, we suggest assessing the effect of *B. infantis* on IL-10 levels, in either the hypothalamus or amygdala. Our study also points at the potential benefit of a more complete understanding of probiotics and their range of effects, particularly when in use with immunologically vulnerable populations.

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Table 1

Number of animals per group

	Hypoxia		Normoxia	
Group	Male	Female	Male	Female
Intervention-Probiotics	6	6	10	8
Intervention-Saline	13	11	9	9

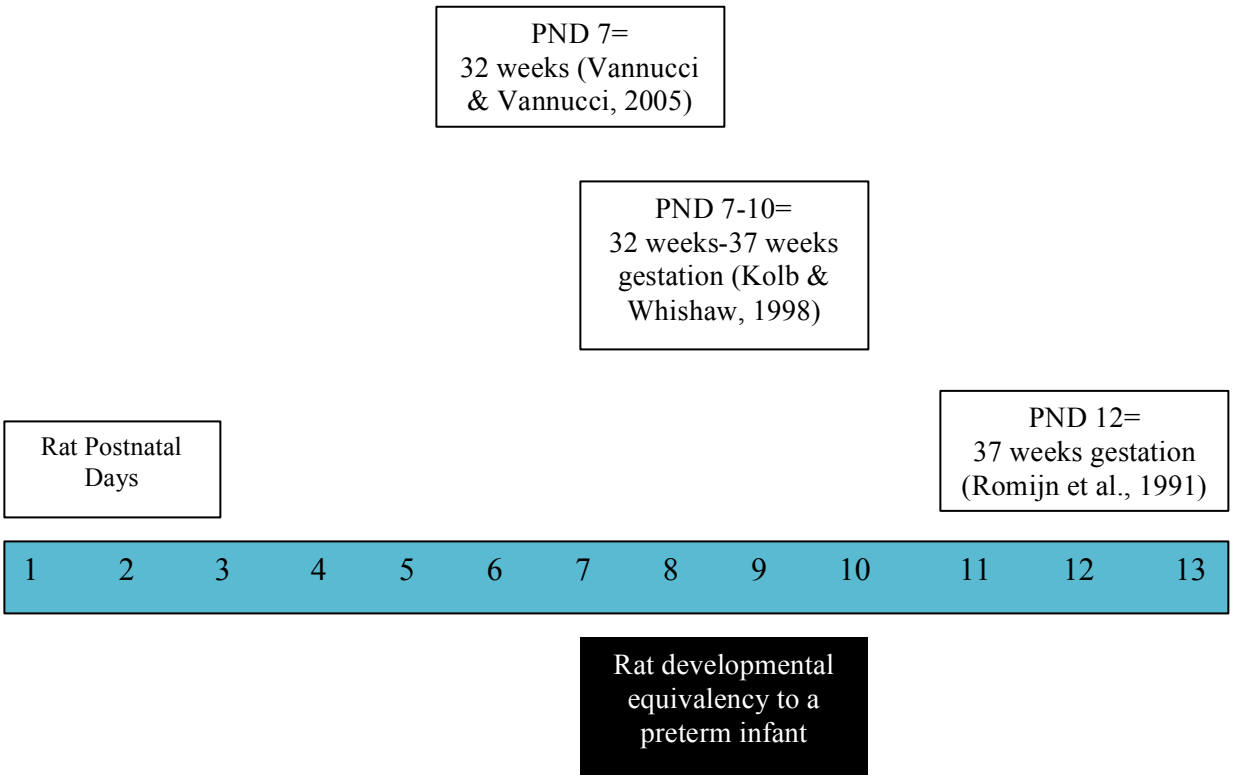
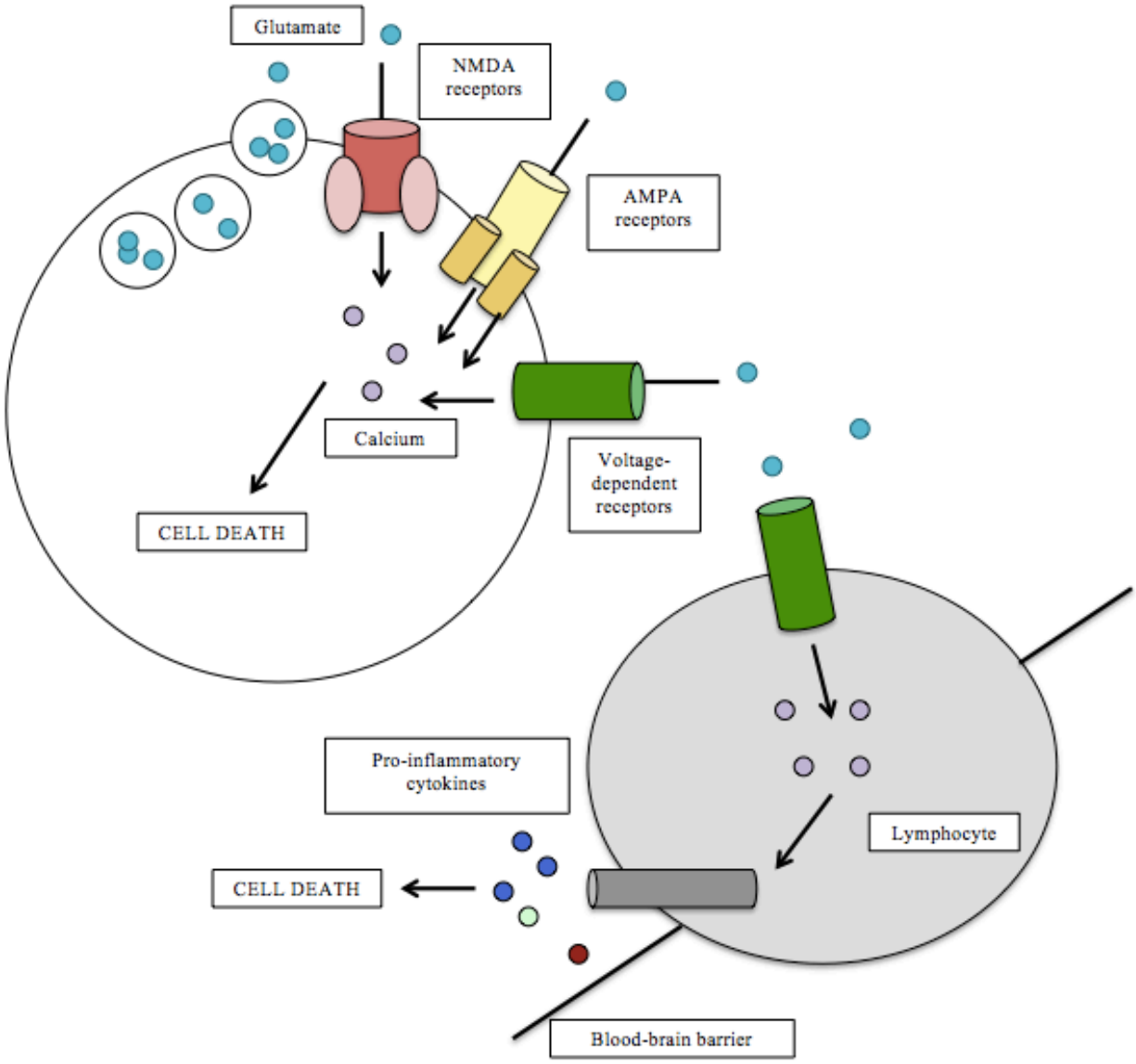


Figure 1. The developmental-equivalency of the rat to a late-gestation preterm infant, based on the definitions of Vannucci & Vannucci (2005), Romijn and colleagues (1991), and Kolb & Whishaw (1998).



F*igure 2*. In the absence of ATP, glutamate is released into the synapse, acting on NMDA, AMPA, and metabotrophic receptors. Once activated, these receptors induce an influx of calcium, which activates mechanism of cell death. Additionally, extracellular glutamate acts on the glutamate receptors of T cells, particularly Th1 cells following HI. This activation induces calcium signalling, resulting in the release of the pro-inflammatory cytokines IL-6, TNF-α and IL-1β, which go on to induce cell death.

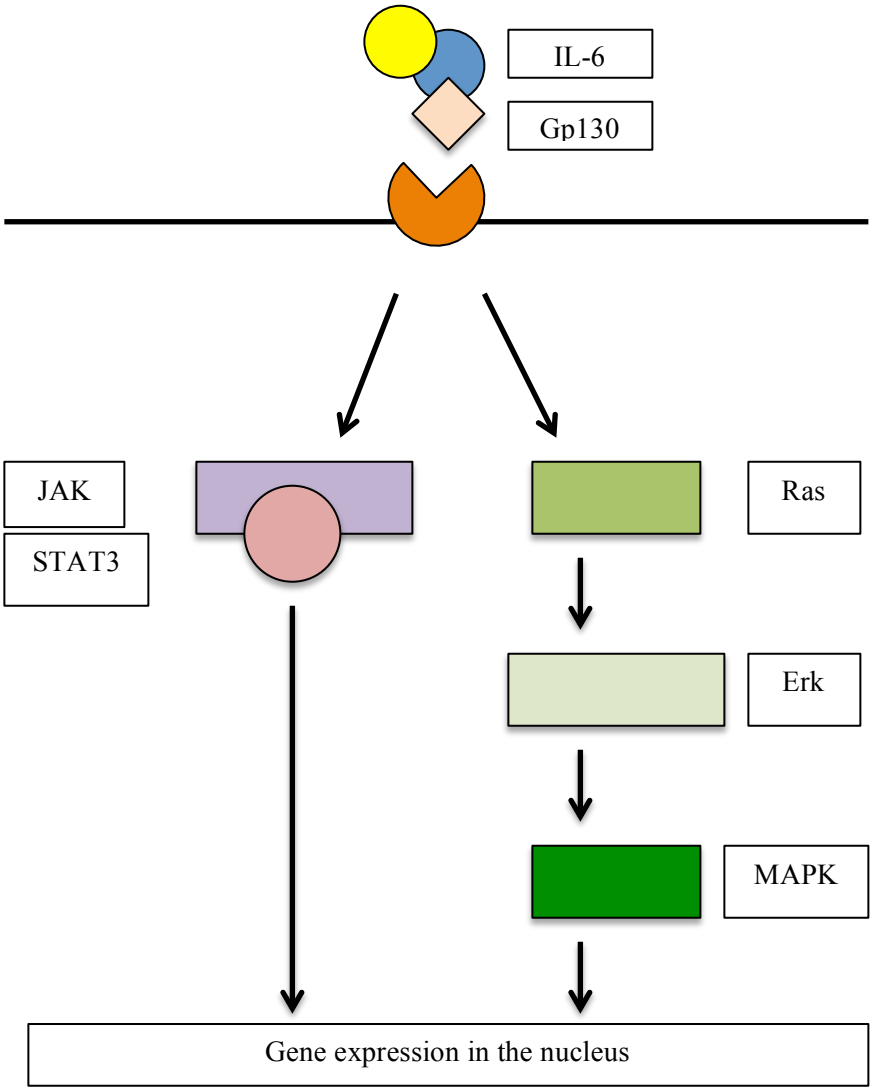


Figure 3. IL-6 effects cell growth through two pathways. Via both the JAK-STAT3 pathway and the Ras-Erk-MAPK pathway, IL-6 can communicate with the nucleus, mediating gene expression.

Figure 4. An example of the gastrointestinal observations when tissue was collected (PND10) from animals exposed to hypoxia and probiotics. The tissue damage, potentially indicative of gastrointestinal inflammation, was found in at least three pups from this group (6 pups in total were examined from this group).

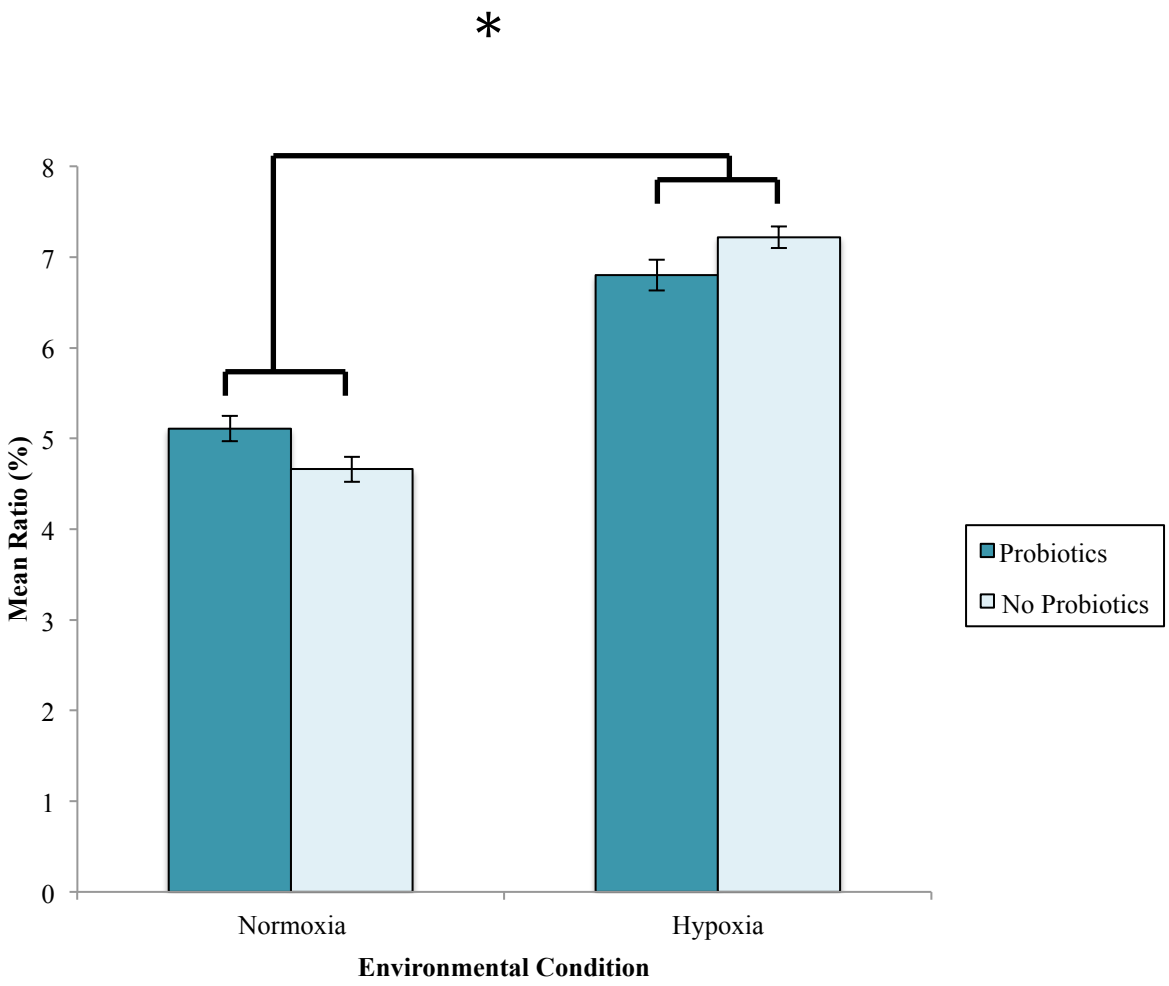


Figure 5. Effect of environmental condition and probiotic administration on brain weight to body weight ratio. Mean (+/-SEM) of all ratios in both the hypoxic (n=24) and normoxic condition (n=36). Mean Ratio (%) is labelled on the Y axis and Experimental Condition is labelled on the X axis. The animals reared in normoxic conditions had lower brain weight to body weight ratios than animals reared in hypoxic conditions. An * indicates $\alpha<0.05$.

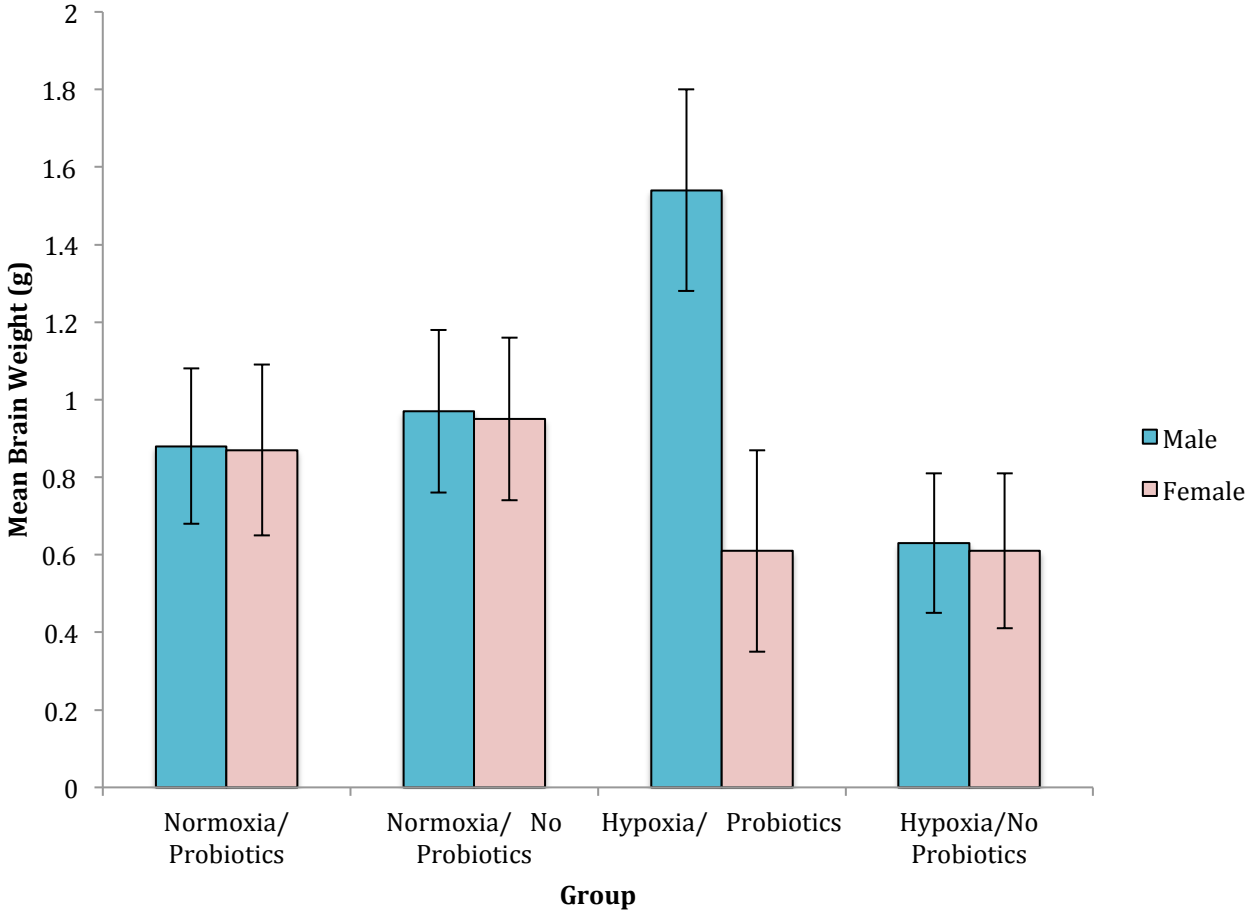


Figure 6. Effect of group and sex on brain weight. Mean (+/-SEM) of all weights in both the hypoxic (n=24) and normoxic condition (n=36). Mean Brain Weight is labelled on the Y axis and Group is labelled on the X axis. There were no significant differences between groups or sexes.

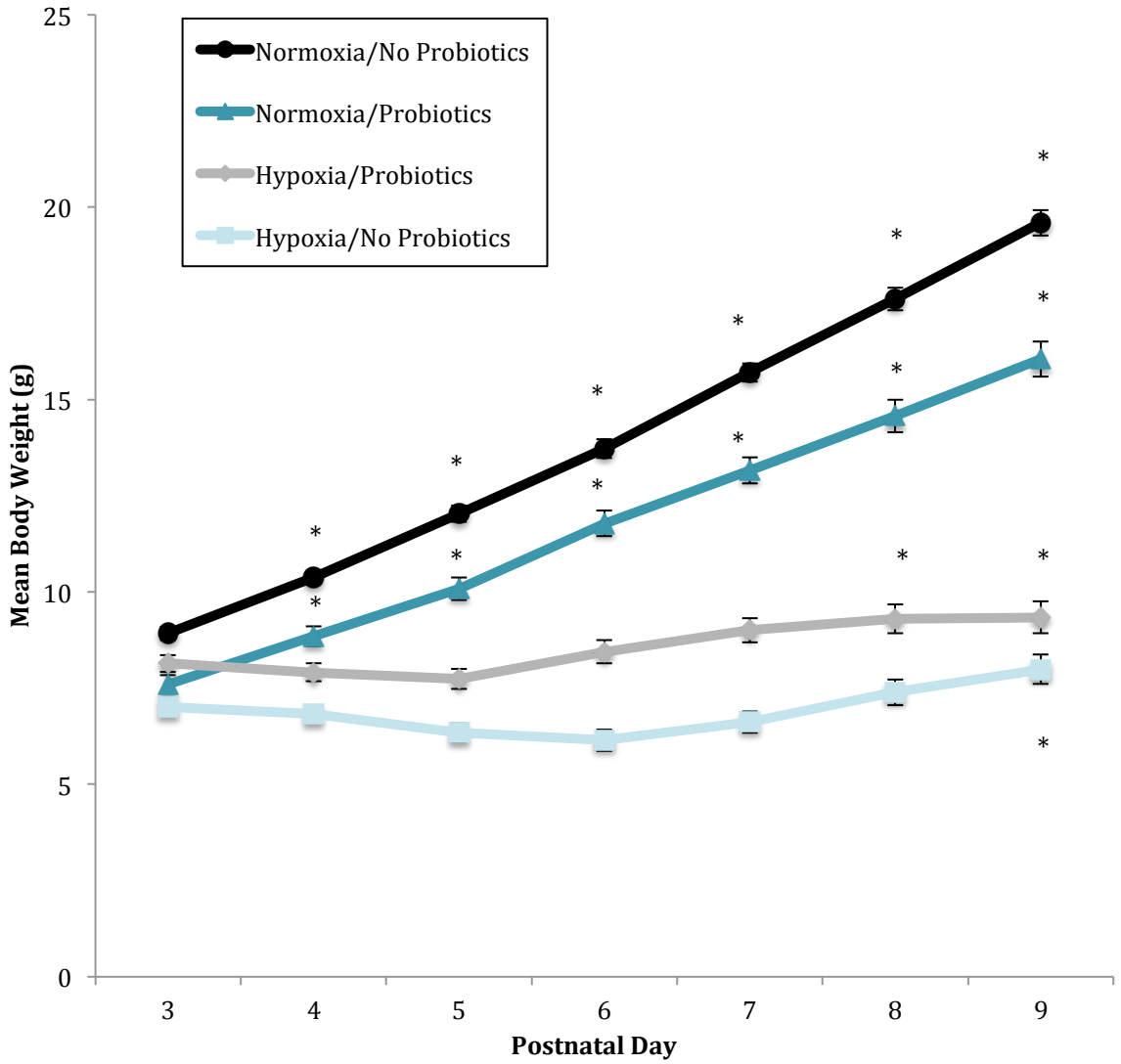


Figure 7. Effect of group and day on body weight. Mean (+/-SEM) of all body weights in the four experimental groups across PND 3-PND 9. Mean Body Weight is labelled on the Y axis and Postnatal Day is labelled on the X axis. Both normoxia groups differed in weight significantly each day following PND 3. Both hypoxia groups demonstrated slower growth, differing from their PND 3 weight at the end only. An * indicates $\alpha<0.05$.

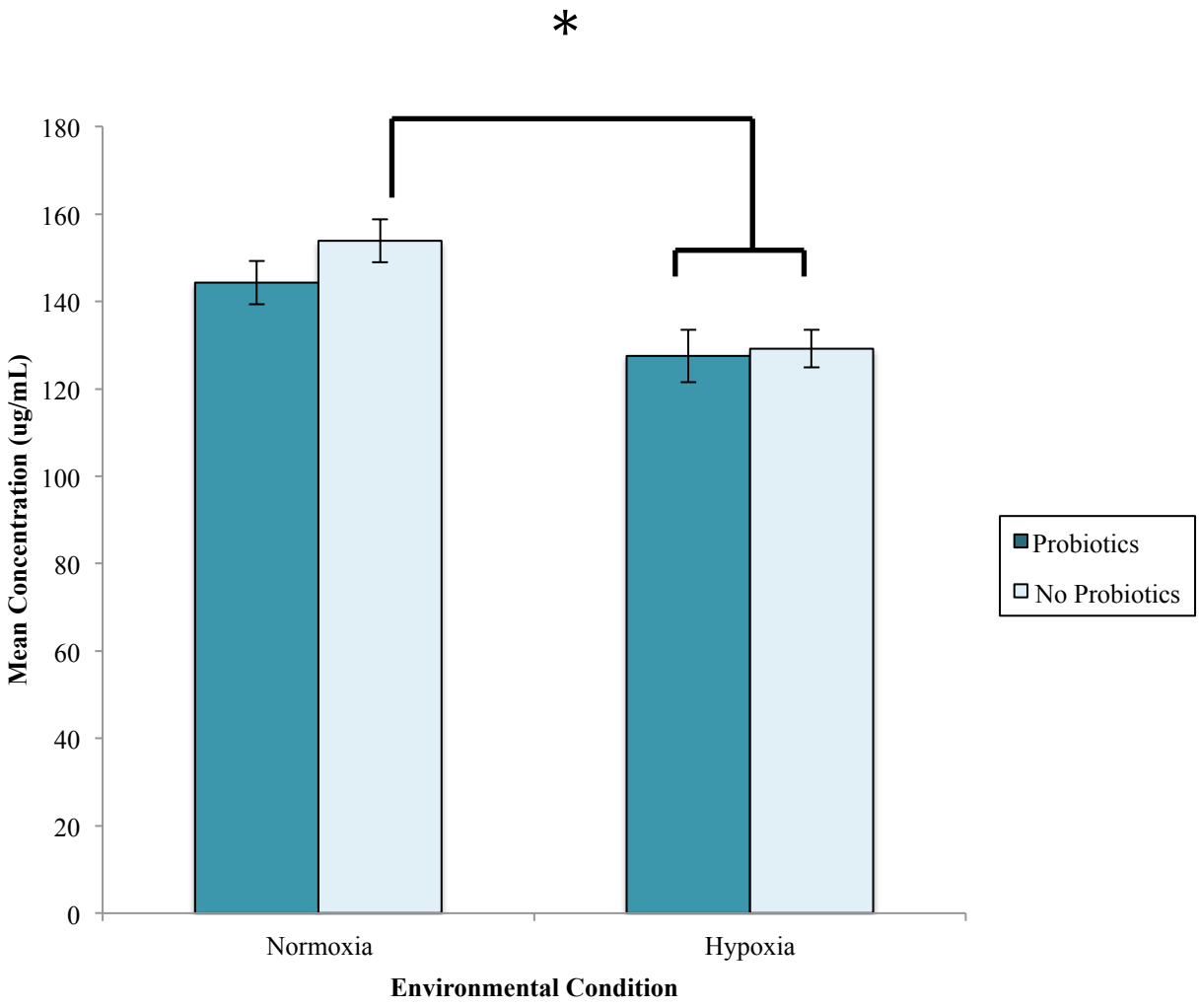


Figure 8. Effect of environmental condition and treatment on hippocampal IL-6 concentration. Mean (+/-SEM) of all concentrations in both the hypoxic (n=24) and normoxic condition (n=36). Mean Concentration is labelled on the Y axis and Experimental Condition is labelled on the X axis. The animals reared in normoxic conditions, that received probiotics, had higher Il-6 levels than other groups of animals. An * indicates $\alpha<0.05$.

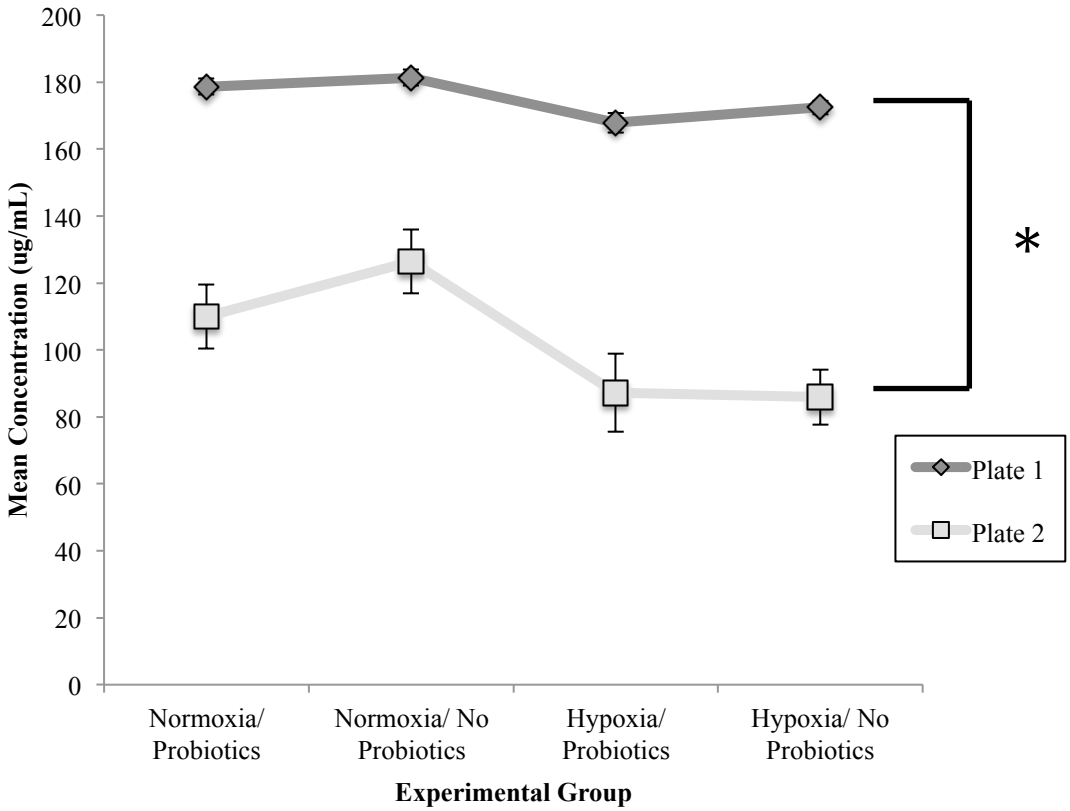


Figure 9. Effect of plate on hippocampal IL-6 concentration. Mean (+/-SEM) of all concentrations in the four experimental groups (n=18, n=18, n=12, n=24, respectively). Mean Concentration is labelled on the Y axis and Experimental Group is labelled on the X axis. All four groups had a significant effect of plate, however, the trend across the groups remained the same across plates. An * indicates $\alpha<0.05$.

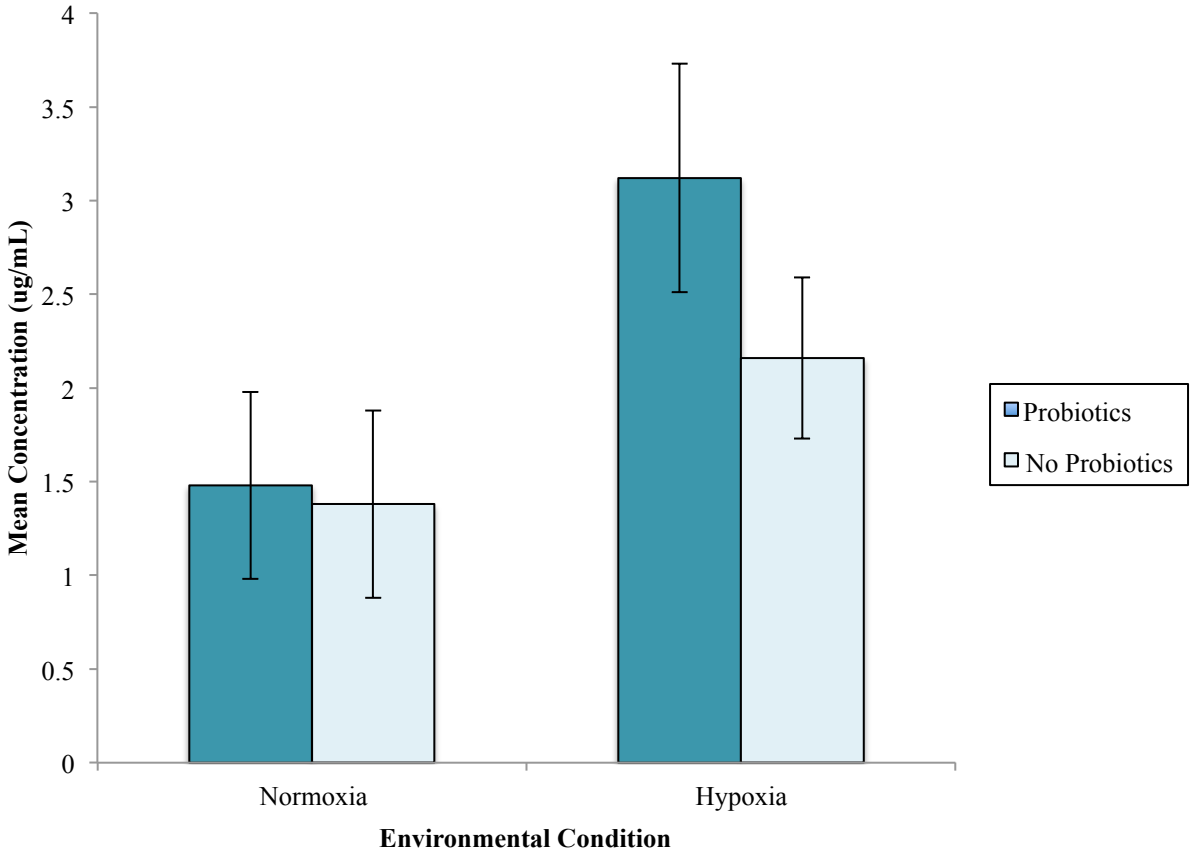


Figure 10.. Effect of environmental condition and treatment on serum corticosterone concentration. Mean number (+/-SEM) of all concentrations in both the hypoxic (n=24) and normoxic condition (n=36). Mean Concentration is labelled on the Y axis and Experimental Condition is labelled on the X axis. There was no significant difference between groups.