Biomarkers of perinatal hypoxia in a rat model

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Abstract

Fetal hypoxia can cause extremely preterm birth with high incidence of brain injury in humans, however, the underlying mechanisms are less understood. In our study, the role hypoxia played on brain development, stress hormones and immune responses in a developing system was examined. Behavioral development was evaluated by seven spontaneous behavioral tests. ELISA was used to measure cortisol and corticosterone concentrations and quantitative RT-PCR was used to quantify gene expression of immune responses after perinatal hypoxia in rats. Hypoxic animals exhibited impaired spontaneous motor behaviors in several tests, including ambulating behavior, strength test and cylinder exploration. Perinatal hypoxia caused increased levels of cortisol and corticosterone. Cortisol levels stayed high, but corticosterone dropped down to control levels five days after perinatal hypoxia. Fourteen out of eighty four genes on the rat innate and adaptive immunity array were altered in our study. Findings demonstrate perinatal hypoxia has negative effects on brain development and can cause the up-regulation of cortisol and corticosterone, as well as the altered expression of immune responses. The immune responses and glucocorticoids are potential biomarkers of perinatal hypoxia. These molecules may be linked to long term cognitive and learning disabilities in preterm birth patients. With the detection of early biomarker levels clinicians may be able to intervene preterm birth at the early stage, consequently leading to a better outcome.

Acronyms

ACI: acute cerebral infarct

AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

ATP: adenosine triphosphate

BBB: brain blood barrier

BDNF: brain-derived neurotrophic factor

CA: Cornu Ammonis

CNS: central nervous system

COX-2: cyclooxygenase-2

DAMPs: damage-associate molecular patterns

DCs: dendritic cells

E: embryonic days

ELISA: Enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

ERK: extracellular signal-regulated kinase

GCRs: cytosolic glucocorticoid receptors

GR: glucocorticoids receptors

GREs: glucocorticoid response elements

HI: hypoxia-ischemia

HMGB1: high-mobility group box 1 protein

ICAM-1: intercellular adhesion molecule-1

IGF: insulin-like growth factor

IκK: I-κB kinase

IL: interlukin

IL-1R1: IL-1 receptor type I

IL-1R2: IL-1 receptor type II

iNOS: inducible nitric oxide synthase

IRAK: interlukine-1 (IL-1) receptor associated kinase

IRF: interferon-β promoter-binding protein

JNK: c-Jun N-terminal kinase

LPS: lipopolysaccharide

MAL: MyD88 adaptor-like protein

MAPK: mitogen activated protein kinase

MCAO: middle cerebral artery occlusion

MR: mineralocorticoid receptors

MyD88: myeloid differentiation primary response gene 88

NF-κB: Nuclear factor kappa light polypeptide gene enhancer in B-cells

NMDA: N-methyl-D-aspartate

NSB: non-specific binging

OD: optical density

PAMPs: pathogen-associated molecular patterns

PND: postnatal day

SARM: sterile α- and armadillo motif-containing protein

TAK: transforming growth factor (TGF)-β-activated kinase

TBI: traumatic brain injury

TIA: transient ischemia attack

Th1: T helper 1

Th2: T helper 2

TIR: Toll-interleukin I receptor

TLRs: Toll-like receptors

TNF-α: tumor necrosis factor-α

TNFR: TNF receptor

TRAF: tumor necrosis factor receptor-associated factor

TRAM: TRIF-related adaptor molecule

TRIF: TIR-domain-containing adaptor protein inducing interferon (IFN)-β-mediated transcription factor

11β-HSD1: 11β-hydroxysteroid dehydrogenase 1

11β-HSD2: 11β-hydroxysteroid dehydrogenase 2

Chapter 1

Effects of hypoxia on glucocorticoids and the immune responses

Hypoxia is relatively common among fetuses and newborns, and leads to significant death and disability in children each year. Reduced oxygen delivery to the developing brain can induce a cascade of downstream effects, which have negative consequences on development in immature brain. The decreased oxygenation and its downstream effects are likely to play major roles in secondary brain injury after the hypoxic event. Among these cascade reactions, many studies have focused on stress hormones (glucocorticoids) (Dong et al., 2011; Guo et al., 2010; Rees et al., 2011) and immune responses (Shalak & Perlman, 2004). A number of studies have shown hypoxia has effects on motor behavior (Grojean, Schroeder, Pourie, Charriaut-Marlangue, Koziel, Desor, Vert, & Daval, 2003; Cai et al., 1999; Golan, Kashtuzki, Hallak, Sorokin, & Huleihel, 2004). The effects of hypoxia occurring during the early third trimester and stress hormones and immune response have not been fully investigated, nor has subsequent behavioral development.

According to the National Center for Health Statistics, fetal hypoxia was among the top 10 causes of neonatal death in the United States in 2004 (Minino et al., 2006). Common risk factors of *in utero* hypoxia include cigarette smoking, drug abuse, anemia, pulmonary disease, hypertension, or high altitude (Zhang, 2005), indicating prenatal hypoxia can be seconday to these factors. Maternal cigarette smoking exposes fetuses to repeated hypoxia, in addition to the toxic effects of chemical compounds in cigarettes, which include benzo(a)pyrene (Stephan-Blanchard et al., 2011). Anemia and pulmonary disease during pregnancy can result in less oxygen transported to fetus. Maternal obesity

and gestational diabetes mellitus may induce chronic hypoxia as a result of vascular structure alteration in the placenta (Li et al., 2013). Any other factors that can cause a compromised intrauterine environment, such as umbilical cord occlusion, uterine artery narrowing, and intrauterine infection, can lead to fetal hypoxia (Rees et al., 2011). Other factors may lead to secondary hypoxia.

Hypoxia can be secondary to other pathophysiological processes. For example, intraventricular hemorrhage, which is a common brain injury in preterm infants that may include the germinal matrix tissue, results in a prolonged decrease of cerebral blood flow with secondary cerebral oxygen deficiency (Volpe et al., 1983; Perlman & Volpe, 1982; Ment et al., 1984). Hypoxia can also be secondary to neonatal pulmonary dysfunction. Preterm infants often have problems with lung maturation (Meisels, Plunkett, Pasick, Stiefel, & Roloff, 1987), which may result in chronic hypoxia after birth. If we cannot eliminate the causes of hypoxia injury, we must learn more about how to intervene should it occur.

Acute hypoxic insults in early-middle gestation can result in the death of Purkinje cells in the cerebellum, pyramidal cells in the hippocampus and cortical neurons (Rees et al., 1999). Acute hypoxia results in neuronal death in the cerebral cortex and striatum in the near term fetal sheep (Loeliger et al., 2003). Chronic fetal oxygen deficiency in arterial blood causes a reduction in brain weight, delayed axonal myelination, and reduced cell numbers in the central nervous system (Rees et al., 2011). In an animal model of chronic hypoxia, enlargement of the lateral ventricles occurs, as a result of reduced growth of neural processes and reduced neuronal numbers in some brain areas (Mallard et al., 1999). Schwartz et al. (2004) also found white matter damage after

chronic neonatal hypoxia. During fetal development, neurogenesis, synapse formation, and neuronal migration are occurring, which makes the brain susceptible to impairment.

Neuroplasticity refers to changes in neural pathways and synapses that are due to changes in behavior, environment and neural processes (Kolb, 1995). The abnormal release of some neurotransmitters, such as glutamate, γ-aminobutyric acid (GABA), acetylcholine, and serotonin, during the pathological process of brain injury induced by hypoxia may disrupt the processes of normal synaptic plasticity. Study by Johnston et al. (2009) found the glutamate receptors, such as N-methyl-d-aspartate (NMDA) channels, can be opened in response to the accumulation of glutamate after neonatal hypoxia-ischemia (HI). This enhanced function of glutamate receptors not only contributes to neuronal injury, but also damages synaptic plasticity (Johnston et al., 2009). Another study focusing on the rat visual cortex after neonatal cerebral hypoxia-ischemia found plasticity mechanisms were impaired after early brain injury, and this impairment was related to the alteration of neuronal development and cortical activation (Failor et al., 2010). Neuroplasticity has different responses to sensory stimulation depending on whether or not the brain has an injury. Kolb and Gibb (2010) found tactile stimulation reduced spine density in intact newborn rats, but increased spine density after frontal or parietal cortical injury in newborn rats. This study suggests brain injury may change neuroplasticity. Hypoxia may impair neuroplasticity and brain development. A number of studies have shown hypoxia has negative effects on both motor behavior and later learning ability (e.g. Cai et al., 1999; Grojean et al., 2003). Both pathophysiologic processes and altered neuroplasticity in the brain after hypoxia may result in motor deficits and cognitive disabilities.

In medicine, a biomarker refers to a molecule that can be measured in a patient, reflects pathology of disease, and becomes a predictive factor for long term outcomes (Woodcock & Morganti-Kossmann, 2013). Biomarkers, such as microRNA, have been widely used in detecting and monitoring cancers (Mar-Aguilar et al., 2014; Xu et al., 2013). microRNA-21 can be used to predict the response in cancer immunotherapy (Wang et al., 2013). Low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides have been used to predict coronary heart disease (Arsenault et al., 2011). The literature provides substantial information on the effects of hypoxia in adults, which is usually secondary to anemia (Hare et al., 2012), hypoperfusion (Dellinger et al., 2013), and obstructive sleep apnea (Murase et al., 2013). The corresponding biomarkers of adult hypxia secondary to anmia and hyperfusion have been also studied in the above literature, such as plasma methemoglobin in hypoxia secondary to anemia (Hare et al., 2012) and lactate in hypoxia secondary to hypoension (Dellinger et al., 2013). Acute mountain sickness occurs when people are in high altitude, which is associated with hypoxa. Julian et al. (2011) investigated the blood biomarkers of acute mountain sicknessand found interleukin-1 receptor agonist (IL-1RA), heat shock protein (HSP)-70 and adrenomedullin were negatively related to acute mountain sickness, but macrophage inflammatory protein-1β was positively related to acute mountain sickness. Biomarkers of prenatal hypoxia and preterm birth, however, are less understood. In the present study, biomarkers of perinatal hypoxia in rats may exist in innate and adaptive genes and glucocorticoids, The levels or expression of the molecules that were significantly altered are potential biomarkers. The biomarkers should be highly correlated with prenatal hypoxia. To move further into a non-invasive domain, they should be

evaluated in plasma and evaluated for whether they predict a long term outcome from exposures to prenatal hypoxia. It would be of great benefit to the patient if an endogenous molecule could be identified to link to prenatal hypoxia or preterm birth and have predictive value with respect to the expected negative consequences of hypoxia, or any good long term outcome that might be predicted. Further, it should provide a link with therapeutic interventions, with markers indicating what interventions might work and what contraindications exists for therapeutic interventions. With the detection of biomarker levels at an early stage, clinicians may be able to intervene preterm birth at the early stage, consequently leading to a better outcome.

Possible mechanisms underlying brain injury after perinatal hypoxia

Hypoxia causes a reduction in oxygen delivery to the brain. Systemic hypoxia can also cause reduced delivery of nutrients such as glucose and amino acids to the brain. A reduction in cerebral perfusion can also contribute to reductions in cerebral oxygen supply due to the negative effects of systemic oxygen deficiency on myocardial function (Lee et al., 2005). Hypoxia may initially be separated into an acute brain injury stage (also known as a primary insult) and a secondary (delayed) brain insult stage at cellular and molecular levels. Different physiological damage may occur in each stage, potentially impacting the brain differently. In the literature, a primary insult and a secondary insult have been defined in several different brain injuries. In neonatal HI, the primary insult is related to the original reduction in cerebral blood flow and oxygen delivery and the primary insult may occur after cerebral oxygeneation and perfusion is restored (Shalak & Perlman, 2004). In perinatal asphyxia, .the primary insult is associated with the duration of oxygen deprivation and the secondary insult relates to re-

oxygenation (Herrera-Marschitz et al., 2014). In cerebral ischemia, cerebral occlusion leads to primary insult and the following reperfusion is related to secondary insult (Kleindienst et al., 2013). The secondary insult is usually related to a cascade of biochemical events following the primary insult (Shalak & Perlman, 2004; Herrera-Marschitz et al., 2014; Kleindienst et al., 2013). No report, however, has clarified the secondary insult after perinatal hypoxia. In our study, the primary insult was related to the period of oxygen deprivation. We assumed a secondary insult would happen during the post-hypoxic period because the secondary insult is associated with re-oxygenation.

Primary insult. The decrease in cerebral blood flow and oxygen delivery immediately triggers a cascade of chain reactions characterized by ATP depletion, anaerobic glycolysis, and metabolic acidosis (Verklan, 2009). Depletion of oxygen results in a switch to anaerobic metabolism in neural cells. Inadequate oxygen also leads to rapid depletion of high-energy phosphate reserves, including ATP, accumulation of lactic acid, and failure to maintain cellular functions (Wyatt et al., 1989). Reduced ATP availability is related to the dysfunction of ATPase systems, particularly the NA^+/ K^+-ATPase and glial-ATPase (reviewed in Shalak & Perlman, 2004). Na^+/K^+-ATPase dysfunction results in transcellular ion pump failure accompanied with depolarization of the cell membrane due to the accumulation of intracellular Na^+. Na^+/K^+-ATPase dysfunction causes intracellular sodium and water accumulation (cytotoxic edema) and/or cell lysis. At the same time, neuronal membrane depolarization induces delivery of excitatory neurotransmitters, specifically glutamate from axon terminals. The major excitatory neurotransmitter, glutamate, tends to accumulate in the intersynaptic and intercellular spaces because of the dysfunction of glial-ATPase, which is an astrocytic

enzyme normally delegated to its reuptake. Glutamate activates specific cell surface receptors, in particular NMDA and AMPA receptors, resulting in depolarization and an intracellular entry of calcium.

Within the cytoplasm, there is an accumulation of free fatty acid that is from the hydrolyzed lipid of the membrane. Oxygen free radicals within the cytoplasm arise from reductive processes within mitochondria. The free fatty acid can be peroxidated by these oxygen free radicals and, as a consequence, produce detrimental materials. Intracellular accumulation of calcium is the result of increased cellular influx, as well as, decreased efflux across the membrane combined with release from the mitochondria and endoplasmic reticulum (ER). In selected neurons, the intracellular calcium induces the production of nitric oxide, a free radical that contributes to tissue injury (Grow & Barks, 2002). To summarize, a combination of the many deleterious effects causes neuronal dysfunction and cell death during the primary insult.

Secondary brain insult. Following resuscitation or removal from the hypoxic environment, normal cerebral oxygenation and perfusion is often restored. During this reperfusion phase, partial recovery of high-energy phosphates occurs. After a latent period of 1 to 2 days, however, a secondary phase of energy failure may develop. Energy failure during the reperfusion phase involves mitochondrial dysfunction secondary to the acute hypoxic insult. Indeed, mitochondria play a central role in determining the fate of the brain cells in the delayed reperfusion phase. Many research studies also suggest inflammatory processes at this point may contribute more to the secondary brain insult than the acute damage (e.g. Palmer, 1995). Overall, during the restoration phase of hypoxia, brain injury is worsened compared to the damage in the acute phase due to

several basic mechanisms: intracellular calcium accumulation, excitatory neurotransmitter release, free radicals, inflammatory cytokines, and bioactive lipid mediators (reviewed in Shalak & Perlman, 2004, Grow & Barks, 2002). Most of these mechanisms are activated during the phase of acute hypoxia, and in the secondary delayed phase these mechanisms continue to be active and contribute to the progression of the brain injury.

Accumulation of intracellular calcium. Calcium is an intracellular second messenger necessary for numerous cellular reactions. During hypoxia-ischemia, there is an increase of calcium influx resulting from stimulation of the N-methyl-D-aspartate (NMDA) receptors of the calcium channel by glutamate (reviewed in Volpe, 2001, Grow & Barks, 2002). Sustained depolarization results in extracellular calcium entering into the cytoplasm. In addition, the exclusion of the calcium is disrupted by energy failure because the sodium-calcium exchanger is one of the ATP-dependent ion exchange pumps. Moreover, calcium can be released into the cytoplasm from the mitochondria and the ER, which normally has sequestered stores of calcium. These alterations in calcium balance lead to increased cytosolic calcium. The increased intracellular calcium can stimulate endocellular enzymes, including protease and phospholipase. Protease destroys neurofilaments and can lead to cytoskeleton damage with breakdown of the cellular body. Phospholipase hydrolyzes phospholipids which are on the membrane and damage the cellular membrane (reviewed in Distefano & Pratico, 2010). Taken together, the accumulation of intracellular calcium after hypoxia-ischemia can cause neurotoxicity and, as a consequence, contributes to irreversible brain injury.

Release of excitatory neurotransmitters. Glutamate is a major excitatory amino acid within brain. Glutamate cannot be degraded, but can be eliminated from the synaptic cleft by uptake transporters that are energy-dependent, and located in neuronal and glial membranes. The action of glutamate is mediated by specific receptors, such as NMDA and α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA). During hypoxia-ischemia (HI), energy failure damages glutamate uptake and results in extracellular accumulation of glutamate and overstimulation of postsynaptic excitatory amino acid receptors, which can produce excitotoxicity. The intracellular consequence of excitatory amino acid receptors over-activation is the increase of intracellular calcium and sodium, which leads to cell swelling (accumulation of intracellular water) and ultimately cell necrosis. Accumulation of intracellular calcium can activate some calcium-dependent endocellular enzymes including protease and phospholipase which can damage essential components of the cell (reviewed in Grow & Barks, 2002). Thus, the release of excitatory neurotransmitters after hypoxia is toxic to neural cells.

Free radical release. Many research studies have shown increased production of free radicals contributes to neonatal HI brain injury (e.g. Bagenholm et al., 1998; Fullerton et al., 1998). A free radical is any atom or molecule with a single unpaired electron in an outer shell. For most biological structures, free radical damage is closely associated with oxidative damage. Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen, such as oxygen ions and peroxides. ROS (e.g. $O_2^{\cdot-}$, H_2O_2, or $HO^{\cdot}$) can be regarded as one type of free radicals (Halliwell, 2012). Reactive oxygen species are produced within the cytoplasm and mitochondria. Under physiological conditions, low concentrations of reactive oxygen species are produced as a

byproduct of mitochondrial electron transport and are destroyed by endogenous antioxidants and scavengers present in the normal brain. During and following HI, the increased levels of reactive oxygen species are the result of increased production and decreased scavenging of these reactive oxygen species (reviewed in Shalak & Perlman, 2004). There are two major sources contributing to the production of free radicals. One of the sources is derived from the breakdown of ATP. Adenosine from ATP becomes xanthine, which can be oxidized into free radicals. Free radicals are also byproducts of prostaglandin synthesis, which is derived from the breakdown of free fatty acids. The free fatty acids can be oxidized by oxygen into free radicals (reviewed in Shalak & Perlman, 2004). Reactive oxygen radicals can damage DNA, protein, and membrane lipids, and can initiate apoptosis (Chan, 2001). Hypoxia can also cause downregulation of antioxidant enzyme genes. Brouwer et al. (2004) found the gene expression of one of the antioxidant enzymes, manganese superoxide dismutase, was down-regulated in hypoxia-exposed crabs. Eventually, the release of reactive oxygen radicals and down-regualtion of antioxidant enzyme can cause cellular death.

Nitric oxide (NO) is a weak free radical and is formed by inducible nitric oxide synthase (iNOS) during the conversion of L-arginine to L-citrulline. Under physiological conditions, nitric oxide can regulate gastrointestinal motility, vasorelaxation, and synaptic neurotransmission. However, NO has deleterious effects on tissue under the conditions of HI. iNOS is up-regulated in neurons and microglia during and following HI and this induction can lead to the production of large quantities of NO. The adverse effects of NO are seen in its interaction with the reactive oxygen radical superoxide anion, which can produce the more reactive and toxic peroxynitrite. NO can induce DNA damage by

inhibiting ribonucleotide reductase for DNA synthesis. NO may also inhibit the glycolytic enzyme and limit ATP formation to decrease the product of APT (Iadecola, 1997). Although NO is a weak free radical, large quantities of NO can also damage neural cells.

Pro-inflammatory cytokines. Several lines of study have shown the immune-inflammatory system is activated after HI (e.g. Bona et al., 1999; McRae et al., 1995; Savman et al., 1998). Cessation of cerebral blood flow leads to energy depletion and cell death, which can trigger immune responses, ultimately leading to cerebral inflammation (e.g. Wang et al., 2007). Cerebral inflammation is characterized by activation of inflammatory cells, mainly microglia/macrophages that accumulate at the site of injury and release inflammatory cytokines and free radicals (Bona et al., 1999; McRae et al., 1995; Zhang et al. 2010). The inflammatory cytokines released include interleukin-1 (IL-1), interleukin-6 (IL-6), transforming growth factor-β (TGF-β), and tumor necrosis factor (TNF)-α (Han et al., 2002). These cytokines may act on neurons, astrocytes, oligodendrocytes, and endothelium to influence brain injury. Inflammatory cytokines have harmful effects through two distinct mechanisms: direct and indirect. The inflammatory cytokines have a direct toxic effect via increased production of inducible nitric oxide synthase, cyclooxygenase and free radical release. The inflammatory cytokines have an indirect toxic effect by inducing glial cells to produce neurotoxic factors, such as excitatory amino acids (Shalak & Perlman, 2004). The pro-inflammatory cytokines contribute to the hypoxic-ischemic brain injury and animal studies have revealed their negative effects.

Studies have shown the broad effect of inflammatory cytokines on brain injuries, such as cerebral ischemia (e.g. Yamasaki et al., 1995; Yang et al., 1998) and neonatal HI (e.g. Savman et al., 1998). Infarct area increased when IL-1-β was intraventricularly administered to adult rats following the induction of focal brain ischemia (Yamasaki et al., 1995). In adult mice, IL-1-β knockouts had smaller infarcts compared to wild types following the induction of transient global cerebral ischemia (Wang et al., 2007). Ischemic brain damage was reduced when TNF-α was inhibited (Yang et al., 1998), whereas ischemic brain damage was increased when recombinant TNF-α protein was administrated after cerebral stroke (Barone et al., 1997). Clinical data also suggests inflammatory cytokines play a role in the pathogenic progression of hypoxic-ischemic brain injury. In human infants, the concentration of IL-6 and IL-8 in cerebral spinal fluid (measured 72 hours after birth) is increased after birth asphyxia compared to controls and the magnitude of the increases correlate with the severity of encephalopathy (Savman et al., 1998). Newborn screening programs demonstrated the full-term infants who had increased levels of a wide array of cytokines were diagnosed later with cerebral palsy (Nelson et al., 1998). Together, these data indicates the inflammatory cytokines are associated with the brain injury and pro-inflammatory cytokines play an important role in the ongoing brain injury after perinatal HI.

Bioactive lipid mediators. Bioactive lipid mediators include arachidonic acid, prostaglandins, leukotrienes, thromboxanes, and platelet-activating factor (PAF). These mediators play a role in cell signaling. Among these bioactive lipid mediators, PAF should be focused on. PAF is produced following membrane damage and is involved in synaptic transmission and transcriptional regulation. High concentrations of PAF or PAF-

like oxidized lipids may contribute to neuronal injury by increasing intracellular calcium concentrations, by stimulating production and release of pro-inflammatory cytokines (Doucet et al., 1992). Bioactive lipid mediators may contribute to the pathogenesis of cerebral ischemic injury by stimulation of inflammation.

Effects of glucocorticoids on brain and behavior

Several cellular and molecular mechanisms may be involved in brain injury after hypoxia. Glucocorticoids and immune system have effects on brain and behavior. Glucocorticoids usually have fast responses, indicating they play a role in primary insult (Velickovic et al., 2012), whereas the immune system is involved in the progression of brain injury (Stridh et al., 2011; Hedtjarn et al., 2004; Kamel & Iadecola, 2012). Excess glucocorticoids and activation of the immune system contribute to hypoxic brain injury.

Hypothalamic-Pituitary-Adrenal axis. The hypothalamic-pituitary-adrenal (HPA) axis consists of three components: the hypothalamus (particularly the periventricular nucleus (PVN)), the anterior pituitary, and the adrenal cortex. Specialized cells in the three tissues can synthesize and secrete their own molecules or hormones that act as the primary signals of the HPA axis. These hormones are corticotropin releasing hormone (CRH), adrenocorticotropin hormone (ACTH), and glucocorticoids hormones. CRH is mainly produced by cells in PVN and it is necessary to stimulate ACTH production and secretion. ACTH is secreted by anterior pituitary and it directly stimulates glucocorticoid synthesis and secretion from cells in the adrenal cortex. CRH and ACTH may not have any other physiological functions except for stimulating secretion of glucocorticoids either directly or indirectly (reviewed in McEwen et al., 1997). Glucocorticoids (cortisol in humans, sheep, and guinea pigs, and corticosterone in

rodents) play diverse roles in development, metabolism, neurobiology, regulation of immune response and many other functions in vertebrate from fish to man (Blum & Master, 2003). Glucocorticoids receptors are found in both the PVN and anterior pituitary and glucocorticoids have a negative feedback effect on CRH and ACTH secretion at the level of the pituitary, hypothalamus, and limbic brain (Keller-Wood & Dallman, 1984). The HPA axis has its own regulatory mechanisms and can keep hormone secretion in dynamic homeostasis.

Glucocorticoid secretion has two modes. In the circadian rhythm, a peak secretion of glucocorticoids occurs at the time of awakening and a nadir secretion occurs during the first few hours of sleep. In the stress response, the secretion of glucocorticoids is phasic (reviewed in McEwen et al., 1997). Both modes operate more or less independently, in which the circadian rhythm is usually not affected by the stress response. Biosynthesis and inactivation of glucocorticoids are commonly completed by hydroxysteroid dehydrogenases (HSD). Two isoforms of hydroxysteroid dehydrogenases have been found: 11β-HSD type 1 (11β-HSD 1) and 11β-HSD type 2 (11β-HSD 2). Usually, 11β-HSD 1 is mainly involved in generating glucocorticoids (cortisol or corticosterone) from inactive cortisone or 11-dehydrocorticosterone, whereas 11β-HSD 2 is mainly implicated in inactivation of cortisol or corticosterone to cortisone or 11-dehydrocorticosterone (Blum & Master, 2003). After glucocorticoid secretion from the adrenal cortex, the metabolism of glucocorticoids depends on the activity of hydroxysteroid dehydrogenases. Glucocorticoids exert their effects by binding to the receptors.

Glucocorticoid receptors and actions of glucocorticoids. The actions of glucocorticoids occur via activation of specific intracellular receptors (Gustafsson et al.,

1987, Schmidt & Litwack, 1982). Two receptor subtypes for glucocorticoids have been found: Type-I receptors or mineralocorticoid receptors (MR), and type II receptors or glucocorticoid receptors (GR) (Arriza et al., 1988; Beaumont & Fanestil, 1983). Glucocorticoids exert their effects through genomic and nongenomic mechanisms and have functions in many physiological and pathological processes.

Both MR and GR are members of the nuclear hormone superfamily of ligand-activated transcription factors and exert genomic effects (reviewed in Stahn & Buttgereit, 2008). Glucocorticoids can easily pass through plasma membranes due to their lipophilic structure, indicating glucocorticoids can go into the cytoplasm to activate genomic mechanisms. These agents bind to the ligand-binding domain of the cytosolic glucocorticoid receptors (GCRs), which can activate these cytosolic glucocorticoids receptors. The glucocorticoid- GCR complex then translocates to the nucleus and this complex binds to DNA-binding sites termed the glucocorticoid response elements (GREs) (Almawi & Melemedjian, 2002). These DNA-binding sites are in the promoter regions of target genes. Depending on the target gene, the glucocorticoid- GCR complex activates or inhibits downstream gene expression. The former is shown by the capacity of the complex to up-regulate the gene transcription of IL-10 and inhibitor of nuclear factor κB (Stahn & Buttgereit, 2008). The latter is evidenced by the glucocorticoid- GCR complex inhibiting gene transcription of IL-2 and other cytokine genes (Mori et al., 1997). The glucocorticoid- GCR complex can interact with other transcription factors to negatively regulate gene transcription. For nuclei in which glucocorticoids have a role, the glucocorticoid- GCR complex can associate with transcription factors (e.g. apolipoprotein 1 transcription factor), which prevents these transcription factors from

exerting their effects on specific DNA sites. Decrease of transcription factor availability results in decrease of transcription of downstream target gene. In addition, the glucocorticoid- GCR complex can reduce target genes expression by binding to specific DNA sites that the transcription factors of these genes bind to (Blum & Master, 2003). Glucocorticoids bind to the receptors to regulate down-stream gene expression.

Glucocorticoids have nongenomic mechanisms of action. High concentration of glucocorticoids can change the properties of biological membranes, especially plasma and mitochondrial membranes (Buttgereit et al., 2004, Buttgereit & Scheffold, 2002). Glucocorticoids can enter into these membranes and change the function of membrane-associated proteins, resulting in increase of lipid peroxidation and membrane permeability. Eventually, glucocorticoids can damage cells by changing the properties of membranes. Nongenomic effects of glucocorticoids are also dependent on the cytosolic glucocorticoids receptors.

Generally, the inactivated ligand-free glucocorticoid receptors associate with other proteins, such as heat-shock protein 90 (Dalman et al., 1991). When glucocorticoids bind to their receptors, proteins that associate with ligand-free glucocorticoid receptors are released. These proteins are thought to be responsible for some of the rapid effects of glucocorticoids (Croxtall et al., 2000). Nongenomic effects of glucocorticoids can be implemented by membrane-bound glucocorticoid receptors. These receptors are expressed in many kinds of cells, such as neuronal membranes (Orchinik et al., 1991), peripheral blood mononuclear cells (Bartholome et al., 2004; Song & Buttgereit, 2006), and T cells (Lowenberg et al., 2006). Glucocorticoids can bind to these membrane-bound

glucocorticoid receptors located on the cell surface to play their role in many physiological or pathological processes.

Glucocorticoids affect fetal development through the receptors MR and GR. The expression of the genes encoding MR and GR are closely related to the impact of fetal glucocorticoid levels. In fetal rat brain, GR mRNA expression is first seen on embryonic day (E) 12.5 and its expression levels increase from that point (Diaz et al., 1998). The expression of MR is present in the fetal rat brain on E 15.5 and maintained at a low level until birth. In the developing human brain, both GR and MR receptor types are expressed in hippocampus between 24 and 34 weeks gestation and the expression levels of GR and MR mRNA do not show large changes over time (Noorlander et al., 2006). The expression of GR and MR is commonly detected at a relatively later time during gestation. The late expression of GR and MR in the brain during gestation is ideal for the receptor types to be associated with the physiological function of glucocorticoids that promote the maturation of organ systems in the body.

Brain development in humans and rats. The development of the brain is characterized by proliferation of neural cells, which requires a relatively long period of time during gestation. In humans, the neural plate initially forms along the dorsal side of the embryo at around 3 weeks gestation. The majority of neurons and glial cells are generated from the neural plate. Between 6 and 18 weeks gestation, major neocortical neurons are generated (reviewed in Uylings, 2000). The generated neurons need to migrate to their destination and self-organize into different layers. This cell migration begins at 8 weeks gestation (Molliver et al., 1973). The neuronal division ends at 20 weeks gestation, whereas, the neuronal migration does not end until birth (Dobbing &

Sands, 1973). There is a restructuring period where new synaptic connections are formed, from gestational age 24 to 28 weeks, and that continues into perinatal periods (Kostovic et al., 2002; Olson et al., 1973; Nobin & Bjorklund, 1973; Mrzljak et al., 1988). The peak time of synapse formation is from 34 weeks of gestation through to 24 months after birth (Bourgeois, 1997; Kostovic et al., 1995; Huttenlocher et al., 1982). In rats, brain development is less mature at birth compared to humans. The neurons are generated mainly from embryonic day 10 until birth (reviewed in Uylings, 2000). The neuronal migration ends around 7 days after birth (reviewed in Kolb, 1995). The peak synapse formation occurs from embryonic day 12 to PND 16 (reviewed in Levitt, 2003). Brain development is clearly different between humans and rats, but glucocorticoids may play similar roles in brain development regardless of species.

The role of glucocorticoids in brain development. Glucocorticoids are important for normal development and maturation of the brain. Neural cell replication, differentiation, and synaptogenesis are completed during the onset of competence of the HPA axis (Miyabo et al., 1980). Meyer (1983) found reduced secretion of glucocorticoids in rats by removal of adrenal tissues resulted in enhanced brain growth, stimulation of glial proliferation and myelinogenesis. Glucocorticoids may initiate terminal maturation, remodel axons and dendrites, and regulate programming cell death.

During early fetal development, the fetus is exposed to very low level of glucocorticoids compared to late gestation due to the placental 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) barriers (Xiong & Zhang, 2012). The 11β-HSD2 can convert active glucocorticoids into their inactive 11-keto metabolites. Approaching term, maternal glucocorticoids continue to increase, whereas the expression of placental 11β-

HSD2 drops significantly, which allows enough glucocorticoids into the fetus for accelerating tissue maturation. The glucocorticoids are increased rapidly later in gestation when the brain is maturating. In humans, programmed cell death, brain restructuring, and synapse formation begins at approximately 24 weeks gestation (reviewed in Levitt, 2003). The peak time for synapse formation begins at gestational age of 34 weeks. In rats, synapse formation begins on embryonic day 12. Brain remodeling and synapse formation are indicative of brain maturation, which may be related to the increased levels of glucocorticoids later in gestation. The increase of glucocorticoids at late gestation occurs in parallel with the expression pattern of GR and MR that are detected late in gestation. The glucocorticoid receptors are detected at E 13 in rats (Diaz, et al., 1998) and at 24-34 weeks gestation in humans (Noorlander et al., 2006). The earliest detection of glucocorticoid receptors is consistent with the time of brain cell apoptosis and reorganization, indicating glucocorticoids are associated with initiation of brain maturation.

Excess glucocorticoids on brain development. Although physiological levels of glucocorticoid are necessary for brain development, excess glucocorticoids have widespread negative effects on brain development. They may impair neural cells and brain structure. For example, the high levels of glucocorticoids may decrease the number of neurons in hippocampus (Uno et al., 1990), and delay the migration of neurons in cerebral cortex and impede normal brain structure organization. Excess glucocorticoids may also affect behaviors, including motor, emotion, and cognition (Matthews, 2000). The excess levels of glucocorticoids may disturb brain development, which can be observed through behavioral abnormalities.

Alterations in brain cell and structure induced by excess glucocorticoids. Animals (e.g. rats and sheep) share many similarities with humans. Studies from animals can give evidence to understand the effects of excess glucocorticoids. Animal studies have shown excess glucocorticoids can damage brain cells (e.g. Uno et al., 1990; Mandyam et al., 2008). Stress and increased glucocorticoids have a major impact on hippocampal cells (De Kloet et al., 1998). After prenatal administration of synthetic glucocorticoids in the fetal rhesus monkey, decreased number of pyramidal neurons in the hippocampal CA regions and granular neurons in the dentate gyrus and degenerated axodendritic synaptic terminals in the CA3 region were found (Uno et al., 1990). Since the increased glucocorticoids were not from stress, but administration of synthetic glucocorticoids in this study, it suggests glucocorticoids can change hippocampal cells. In the long term, glucocorticoids may have harmful effects on brain cells. Mandyam et al. (2008) evaluated cell proliferation, cell death and neurogenesis in adult offspring of pregnant rats exposed to a stressor (a stimulus can cause stress). The authors found decreased proliferating cells, increased cell death, and reduced neurogenesis in female rats, suggesting that prenatal stress or increased glucocorticoids may influence brain structure through to adulthood. The deleterious effects of excess glucocorticoids can be also found in sheep research. Prenatal glucocorticoid administration delayed both astrocyte and vasculature maturation in corpus callosum in sheep (Huang et al., 2001). Another study found excess glucocorticoids in fetal sheep delayed myelination in multiple brain regions such as the internal capsule, centrum semiovale, the superficial white matter, and corpus callosum (Antonow-Schlorke et al., 2009). These studies collectively suggest the increased levels

of glucocorticoids during the prenatal period influence brain development extensively across cell type (e.g. neural precursor cells, neurons, and astrocytes) and within many brain regions (e.g. hippocampus, corpus callosum, internal capsule, centrum semiovale, and the superficial white matter). The effects of increased glucocorticoids are harmful on neural cells across the brain. The altered number of neural cells may change the volume, shape, or size of the brain, as a result of the alteration of brain structure.

Excess glucocorticoids were shown to retard the radial migration of post-mitotic neurons during the development of cerebral cortex (Fukumoto et al., 2009). Fukumoto et al. (2009) identified Caldesmon, which is an actin regulatory protein, as the glucocorticoids' main target. Caldesmon negatively controls the function of myosin II and leads to changes in cell shape and cellular migration. The up-regulation of Caldesmon induced by the administration of glucocorticoids impairs radial migration of neurons during cortical development. The impairment of radial migration of neurons induced by excess glucocorticoids may alter the brain structure directly and severely. Together, the increased levels of glucocorticoids, from both endogenous and exogenous resources, may impair both the brain cells and structure.

It is challenging to find structural changes in human brain associated with fetal stress due to the challenge of obtaining post mortem tissue. There are some human studies that reveal brain structural changes by using MRI scans (Buss et al., 2010; Buss et al., 2007). A prospective human study showed a decrease of gray matter in young children was related to pregnancy anxiety and stress during early mid-gestation (Buss et al., 2010). Several brain regions in young children were affected after prenatal stress, such as the prefrontal cortex, the premotor cortex, the medial temporal lobe, the lateral

temporal cortex, the postcentral gyrus and cerebellum. Another human study found the reduction of hippocampal volume was associated with prenatal stress in adult women (Buss et al., 2007). In summary, excess glucocorticoids exposure during fetal period causes decrease of gray matter and reduction of hippocampal volume, which may render functional and behavioral impairment.

Alterations in behavior induced by prenatal stress or excess glucocorticoids. Increasing evidence from human studies indicates children who have experienced prenatal stress have a delay in walking, speech, and toilet training and signs of emotional disturbance (e.g. Ward, 1991; Papousek and von Hofacker, 1998). There is also a positive correlation between gestational stress and incidence of neuropsychological disorders, such as hyperactivity-attention deficit disorder (Clements, 1992), Tourette syndrome (Leckman et al., 1990), and autism (Angelidou et al., 2012). In addition, prenatal exposure to stress or stress hormones are associated with various kinds of psychological and/or mental dysfunction such as schizophrenia (Van Os & Selten, 1998; Tsuang, 2000), depression (Van den Bergh et al., 2008), and an impaired stress response in offspring (Watson et al. 1999). Watson et al. (1999) reported a greater depression response among offspring who were exposed to maternal stress. Prenatal exposure to maternal anxiety at 12-22 weeks of gestation was consistently associated with depressive symptoms in adult offspring (Van den Bergh et al., 2008). During the first and second trimester, exposure of prenatal stress results in higher incidence of schizophrenic symptoms in offspring (Van Os & Selten, 1998). Imaging studies also found structural abnormality in schizophrenic subjects. Schizophrenic subjects showed enlarged cerebral ventricles with reduced volume of the temporal and frontal lobes, hippocampus, amygdala, basal ganglia and

thalamus (Tsuang, 2000), which were the structural basis of behavioral abnormalities. Prenatal stress and anxiety retard infant cognitive and motor development, but this association may be time-dependent (Davis et al., 2007; Huizink et al., 2003). Prenatal stress early in gestation results in retardation of mental and psychomotor development, whereas, exposure of prenatal stress late in gestation results in accelerated mental development (Davis & Sandman, 2010). Taken together, the evidence suggests prenatal stress can increase the incidence of neuropsychological disorders and mental dysfunction in the offspring. Prenatal stress may not always be negative to motor and cognitive functions, as exposure late in gestation in humans may also have positive effects, but the role of prenatal stress in behavior and cognition is still unclear.

Studies in experimental animals present further evidence for understanding the effect of prenatal stress or excess glucocorticoids on behavior. In rats, prenatal stress can cause depression of sexual activity in male offspring (Dorner et al., 1983), indicating prenatal stress may have a long term effect and influence reproductive behavior. The young rat offspring exposed to prenatal stress showed a reduced tendency for social interaction with other rats (Ward and Stehm, 1991), suggesting prenatal stress may impair social behavior in rats. One study in Rhesus monkeys showed the offspring of monkeys who were exposed to maternal stress during mid-late gestation had less play and exhibited exploration behavior and more anxious behavior when these monkeys were in a novel environment (Clarke and Schneider, 1993). The data indicate the high levels of glucocorticoids, which resulted from maternal stress, may contribute or cause psychiatric disorders.

Keshet and Weinstock (1995) used consumption of sweet substances (e.g. saccharin) to evaluate depressive behavior in rats. The authors found there was a decrease in consumption of sweet substances in female rats with prenatal stress. The female rats normally show an increase in the consumption of sweet substance compared to males, indicating that female offspring with prenatal stress may have a deficit in pleasure or depressive-like behavior. Moreover, offspring of prenatally stressed rats showed abnormal behavior in those tests used to evaluate depression in animals, such as reduced exploratory behavior in an open field and elevate plus-maze, and increased immobility time in a forced swimming test (Alonao et al., 2000; Estanislau & Morato, 2005). In addition to prenatal stress, prenatal glucocorticoids administration in rats also leads to an increase of anxiety-like and depressive-like behavior and these rats have similar performance in the open filed, elevate plus-maze, and forced swimming test (Welberg et al., 2001). Hauser et al. (2009) also found administration of synthetic glucocorticoids in pregnant rats was associated with impaired spatial memory in water maze by the offspring, suggesting prenatal excess glucocorticoids exposure also leads to abnormal cognitive activity in offspring.

Maternal or fetal stress can cause alteration of brain structure and behavior, but this alteration is related to the timing of exposure to stress. Buss et al. (2010) found a decrease of gray matter volume in young children was related to pregnancy anxiety and stress at 19 weeks gestation, whereas pregnancy anxiety at 25 and 31 weeks gestation was not associated with a reduction in grey matter volume. Exposure to elevated levels of glucocorticoids early in gestation was associated with slower motor and mental development at 12 months after birth, but elevated maternal levels of glucocorticoids late

in gestation were associated with increased development during the first postnatal year (Davis & Sandman, 2010). These studies indicate fetal stress might have positive effects on development in offspring depending on the degree of stress and the timing of exposure. Perinatal period in rats may be comparable to human's early third trimester. Although Buss et al. (2010) found beneficial effects of fetal stress on behavior in humans, perinatal stress in rats may not have positive effects on behavioral performance. The fetal stress or maternal stress, accompanied with excess secretion of glucocorticoids, may have two opposing effects on brain and behavioral development. The effects of glucocorticoids, beneficial or destructive, may depend on the time when stress occurs during pregnancy and the severity of stress. A better understanding of the functions of glucocorticoids will allow discoveries of effective interventions during and following fetal stress and avoid the subsequent impaired brain development.

Immune system and brain injury

The central nervous system (CNS) was understood as an immune-privileged system for decades due to the lack of lymphatic drainage, minimal permeability of the blood-brain-barrier, and absence of immune response (Pachter et al., 2003). Extensive work in the last decade has shown there is a specialized intrinsic immune system in the CNS (Rivest, 2009) and the CNS has its own immunoregulatory cells including endothelial cells, microglia, astrocytes, and oligodendrocytes. Under physiological conditions, these cells may not be immunologically related and they play an important role in maintaining homeostasis including protecting and nourishing the brain. The glia (notably, microglia and astrocyte) can present antigens, regulate production and uptake of excitotoxins (for example glutamate), and secrete neurotrophic factors in order to nurture

and protect the CNS (Gendelman, 2002). In addition, Yirmiya and Goshen (2011) reviewed under normal steady-state, the immune system (especially, inflammatory cytokines) played a critical role in learning, memory, neural plasticity and neurogenesis. Deficiency of cytokine signaling results in impaired functioning of learning, memory, neural plasticity and neurogenesis (Yirmiya & Goshen 2011). In addition to the physiological function, mounting evidence also indicates the immune system has a key role in brain injury. The immune responses contribute to most types of brain injury, such as traumatic brain injury (Chen et al., 2011), stroke (Liu et al., 2012), neurodegenerative diseases (Pradhan & Andreasson 2013), and even some mental disorders (Lavretsky & Newhouse, 2012). Glial cells, principally microglia, secrete potentially toxic factors, including pro-inflammatory cytokines, nitric oxide, platelet activating factor, chemokines, excitatory amino acid, and free radicals (Gendelman, 2002). Immune responses occur as a reaction to the pathological processes and worsen brain damage. On the contrary, many studies also find inflammation-associated pathways benefit brain injury (Vexler & Yenari, 2009; Madinier et al., 2009; Parent et al., 2002; Qiu et al., 2007). The immune system plays a dual role, protective and destructive, in the pathophysiological processes.

Components of immune system. The innate immune system is known as a non-specific immune system. Innate immunity also lacks memory (i.e. no enhanced response upon secondary exposure) and it is thought to provide an initial means of defense (McEwen et al., 1997). The cells involved in innate immune responses are myeloid and cytolytic cells, including natural killer cells, mast cells, eosinophils, basophils, macrophages (microglia in the CNS), and neutrophils. These cells do not require prior

activation to provide an important first line of defense during the early stage of an immune response. Pattern-recognition receptors are presented on these innate immune cells such as the Toll-like receptors (TLRs) (Bellavance & Rivest, 2012). Pattern-recognition receptors can recognize a selective pattern referred to as pathogen-associated molecular patterns (PAMPs) or damage-associate molecular patterns (DAMPs). The innate immune cells recognize "non-self" or foreign agents by pattern-recognition receptors recognizing specific patterns, such as PAMPs and DAMPs.

In contrast to innate immunity, adaptive immunity has specificity in response to a specific pathogen or antigen and it can distinguish self from non-self (McEwen et al., 1997). Another characteristic of adaptive immunity is that it has the ability to establish immunologic memory and to enhance immune response upon secondary exposure. The cells mediating adaptive immunity are T and B lymphocytes (Kincade, 1987; VonBoehmer, 1995). T cells include T helper 1 (Th1) and T helper 2 (Th2), which help or induce responses, and cytolytic T lymphocytes, which mediate direct break down of target cells. B lymphocytes can produce antibodies. Both innate and adaptive immune systems can react to the pathological processes in the brain.

Innate immune system in the brain. It has been documented that the innate immune responses are recruited in the brain (Nguyen et al., 2002; Lehnardt, 2010). In fact, the brain shows a well-organized innate immune reaction in response to infectious agents and cerebral injury (Nguyen et al., 2002; Kielian, 2006). Microglial cells are thought to be the main cell type of the innate immune system in the brain. The neurovascular unit including microglia, astrocytes, and endothelial cells constitutes a dynamic interface contributing actively in CNS innate immunity (Hermann and Elali,

2012). In case of infections, trauma, or other pathological conditions, the immune cells in the brain recognize foreign agents or damaged tissue by small patterns (PAMPs and DAMPs) and then produce innate immune responses. These patterns include proteins from bacterial membranes such as peptidoglycans, intracellular proteins including heat shock proteins, and nonprotein products such as ATP and nucleic acid fragment (Kumar et al., 2011). Both PAMPs and DAMPs can be recognized by pattern recognition receptors that include TLRs, Nod-like receptors, and R1G1-like receptors. Of these receptors, researchers are most interested in TLRs. Microglial cells have been heavily studied and it has been found they express these pattern recognition receptors (Hanamsagar et al., 2012). The engagement of such receptors results in the activation of specific pathways (e.g. TLR2 pathway and TLR4 pathway) and the release of specific cytokines that play a role in the processes of brain injury. The endpoint of the innate immune system is the release of cytokines and the introduction of phagocytosis by microglial cells. In summary, the innate immune system is active in the brain and plays an important role in brain injury.

Adaptive immune system in brain. Good examples to describe the existence of adaptive immunity in brain are the autoimmune diseases of the CNS, such as multiple sclerosis (e.g. Wraith & Nicholson, 2012). During the pathogenesis of multiple sclerosis, infiltrated T and B cells regard myelin as an antigen and insult it, resulting in demyelination and neurological dysfunction (Minagar and Alexander, 2003; von Budingen, et al., 2012). T and B lymphocytes are the main cell types of the adaptive immune system and cannot be produced in the CNS. T and B lymphocytes are produced in the bone marrow, circulate in the peripheral blood flow, and can enter into the brain by

specific mechanisms under some pathological conditions. In multiple sclerosis, focal lymphocyte can infiltrate into CNS parenchyma due to brain blood barrier (BBB) dysfunction (Cristante et al., 2013; Compston and Coles, 2008). T lymphocytes usually circulate in cerebral spinal fluid under normal conditions (Engelhardt et al., 2005). Activated lymphocytes make contact with the BBB and cross the barrier by diapedesis, which is one kind of leukocyte extravasation (Yednock et al., 1992). If an antigen is seen, or immunological reactions are activated, these activated lymphocytes can be drawn into a specific site, and then collectively breach the glia limitans to infiltrate the parenchyma. This progress of infiltration is regulated by chemokines and their receptors (Wraith & Nicholson, 2012). Dendritic cells (DCs) are a group of antigen presenting cells and they are the most efficient stimulators of T-lymphocytes. DCs act as messengers between the innate and adaptive immunity. DCs have recently been found in the meninges and choroid plexus of healthy mouse brain (Anandasabapathy et al., 2011). In the inflamed CNS, DCs can cross the BBB by the regulation of adhesion molecules, chemokines and growth factors (Clarkson et al., 2012), accumulate in the CNS, and result in amplification of immune responses (Lehman et al., 1992; Bailey et al., 2007; Kerr et al., 2008). Under inflamed condition, DCs can go into the brain and play an important role in presenting antigens during inflammation. Although the immune cells of adaptive immune system are from the blood, they can infiltrate the brain under inflammation.

Kamel and Iadecola (2012) reported adaptive immunity is involved in the pathological process of stoke. After stoke, the number of antigen-presenting cells (e.g. DCs) in the brain is increased and help brain antigen present to lymphocytes. This antigen presentation results in the production of antibodies against brain antigens and T

cells sensitized to brain antigens. Administration of myelin antigens in stroke models benefits the development of immune tolerance and reduction of brain injury during the subsequent stroke damage. This sequence indicates adaptive immunity is involved in the progression of stroke and that modulating it may be beneficial. Although further research will be required to clarify the precise role of adaptive immunity in brain injury, it cannot be ruled out that a long-lasting autoimmune response to brain antigens can result in damage to the brain.

Toll-like receptor pathway. The immune system plays a role in brain injury by specific pathways, such as Toll-like receptor pathways. Activation of pattern recognition receptors is a key-initiating event of innate immune responses. TLRs are one of the groups of the pattern recognition receptors expressed in the CNS. Numerous data indicate innate immune function of microglial cells strongly depends on TLR signaling (Sierra et al., 2008) and TLRs play a major role in both infectious and non-infectious CNS diseases (Akira et al., 2001; Akira et al., 2006; Kawai & Akira, 2010). So far, 10 and 12 functional TLRs have been identified in humans and mice, respectively, with TLR1- TLR9 being conserved in both species (Akira et al., 2006). TLRs are type I transmembrane proteins, which contain an extracellular leucine-rich repeats domain and an intracellular Toll-interleukin I receptor (TIR) domain. The leucine-rich repeats in the extracellular domain mediate the recognition of PMAPs or DMAPs, and the intracellular TIR domain recruits adaptor protein. The recruitment of the adaptor protein is necessary for downstream signal transduction (Kenny et al., 2008, West et al., 2006). In general, TLRs are expressed on antigen-presenting cells such as B cells, dendritic cells, monocytes, and microglia in the CNS. In addition, TLRs are also expressed on the endothelium and on

cells within the brain parenchyma such as astrocytes, oligodendrocytes, and neurons (Marsh et al., 2009; Carty & Bowie, 2011). Microglial cells and astrocytes express most TLRs 1-9, but only specific TLRs or TLR stimulation can make these cells generate cytokines (Marsh et al., 2009; Arumugam et al., 2009). The stimulation of TLRs 2, 3, 4 activates microglia to generate cytokine and chemokine responses, yet only TLR3 stimulation activates astrocytes to generate minor immune responses (reviewed in Wang et al, 2011). Oligodendrocytes express TLR2 and 3, cerebral endothelial cells constitutively express TLRs 2, 4, and 9, and neurons express TLRs 2, 3, 4, 8, and 9 (Arumugam et al., 2009). Therefore, TLRs are broadly located on the immune regulatory cells in the CNS to regulate innate immunity.

Ligands of TLRs can be from exogenous or foreign pathogens, which are related to infection, and endogenous or host-derived molecules, which are associated with brain injury (Wang et al., 2011). The exogenous ligands are usually microbial components such as lipids, lipoproteins, proteins, or nucleic acids. The endogenous ligands are either molecules released from damaged cells or extracellular matrix breakdown products. These endogenous ligands include fibrinogen, heat shock proteins, saturated fatty acids, or high-mobility group box 1 protein (HMGB1). For example, TLR4 can recognize lipopolysaccharide (LPS), which is from gram-negative bacteria (Mallard et al., 2009), and HMGB1, which is a chromatin-associated protein and released from damaged cells and immune cells (Klune et al., 2008). The above literature indicates TLRs can recognize both exogenous and endogenous ligands.

Generally, a ligand binding with TLR causes the initiation of intracellular signaling pathways and lead to the activation of transcription factors and the generation

of cytokines and chemokines (Takeda & Akira, 2005). In TLR pathways, five adaptors are required to be recruited, including myeloid differentiation primary response gene 88 (MyD88), MyD88 adaptor-like protein (MAL), TIR-domain-containing adaptor protein inducing interferon (IFN)-β-mediated transcription factor (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α- and armadillo motif-containing protein (SARM) (O'Neill & Bowie, 2007; Kenny et al., 2008, West et al., 2006). The majority of TLRs utilize MyD88 to initiate intracellular signaling. TLR3 utilizes TRIF adaptor and TLR4 require both MyD88 and TRIF adaptors to initiate downstream signaling. Recruitment of MyD88 to the activated receptors facilitates to phosphorylate interlukin-1 (IL-1) receptor associated kinase (IRAK)-1. Phosphorylated IRAK-1 activates tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 can then activate protein kinase C, extracellular signal-regulated kinase (ERK)-1/2, and transforming growth factor (TGF)-β-activated kinase 1 (TAK1). TAK1 is able to phosphorylate mitogen activated protein kinase (MAPK) p38, c-Jun N-terminal kinase (JNK), and I-κB kinase (IκK). The phosphorylated IκK can activate transcription factors, nuclear factor kappa light polypeptide gene enhancer in B-cells (NF-κB), interferon-β promoter-binding protein (IRF) 1, and IRF7. These activated transcription factors can initiate expression of downstream genes that are pro-inflammatory cytokines (e. g. IL-1, TNF-α, IL-6) (Takeda & Akira, 2004; Gill et al., 2010). The TLR3 signaling pathway is mediated by the TRIF adaptor. In this pathway, TRAF3 can be activated. The activation of TRAF3 can initiate a series of kinases, and eventually activate transcription factors IRF3 and IRF7. These transcription factors can activate transcription of downstream genes associated with this pathway (Gill et al., 2010; Takeda & Akira, 2004). In summary, TLRs initiate their

specific intracellular signaling pathways to regulate downstream gene expression (e.g. cytokines).

TLRs in brain injury. Accumulating evidence shows TLRs are involved in brain injury, especially in stroke and neonatal HI. Although nine functional TLRs have been identified in both humans and mice, TLR4 and TLR2 are the most studied, suggesting these two receptors may have a close relationship with brain injury. Numerous studies have reported TLR4 is involved in adult focal cerebral ischemia. Animal and human studies have confirmed that *TLR4* mRNA is up-regulated after cerebral ischemia (Yang et al., 2008; Tang et al., 2007). In human studies, Yang et al. (2008) reported TLR4 expression positively correlated with cytokine levels and stroke severity in patients. TLR4 expression was significantly increased in the acute cerebral infarct (ACI) group compared to the transient ischemia attack (TIA) and the control groups, and was also significantly increased in the TIA group compared to the control group. These results suggested that there was a positive correlation between TLR4 expression and the severity of stroke. The cytokine levels of TNF-α and IL-6 in the ACI group were significantly higher than the TIA and the control group, and also significantly higher in the TIA group than the control group. Correlation analysis also showed a positive correlation between TLR4 expression and the levels of TNF-α and IL-6. TLR4 expression is associated with the levels of cytokines and stroke severity.

Mice deficient in TLR4 showed less infarct size than wild-type mice after the use of different models of cerebral ischemic stroke, such as permanent occlusion of middle cerebral artery (Caso et al., 2007, Cao et al., 2007; Caso et al., 2008), and transient occlusion of the middle cerebral artery (Tang et al., 2007; Kilic et al., 2008). TLR4

deficient mice were subjected to permanent occlusion of middle cerebral artery and exhibited less neurological deficits and reduced edema, as well as reduced levels of pro-inflammatory cytokines such as TNF-α and IL-6 compared to wild-type mice (Cao et al., 2007; Caso et al., 2007; Caso et al., 2008). Animal studies also show the correlation between TLR4 and neonatal brain injury. LPS can be recognized by TLR4. Administration of LPS, directly into the developing brain of mice and rats, causes white matter damage (Lehnardt et al., 2002; Pang et al., 2003) and systemic administration of LPS induced white matter lesions in fetal sheep (Mallard et al., 2003) and newborn kittens (Hagberg et al., 2002). One *in vitro* study by Lehnardt et al. (2002) found deletion of the TLR4 gene prevented LPS-induced oligodendrocyte death. Several studies have shown administration of LPS makes the developing rat and mouse brain sensitize to subsequent hypoxia-ischemia (Eklind et al., 2001; Eklind et al., 2005; Wang et al., 2007a; Wang et al., 2007b). Data also suggest that these effects of LPS are mediated through the TLR adaptor protein MyD88 (Wang et al., 2009). However, TLR4 or MyD88 deficiency does not confer protection from HI in neonatal mice (Mallard et al., 2009). Taken together, TLR4 is one of the pattern recognition receptors expressed in the CNS, which regulates innate immunity such as the production of pro-inflammatory cytokines. Moreover, the TLR4 pathway plays a more important role in adult brain injury compared to injury in the developing brain.

Similarly to TLR4, TLR2 is another pattern recognition receptor playing a role in cerebral ischemic injury. Mice studies showed cerebral ischemia resulted in the up-regulation of *TLR2* mRNA in lesion-associated microglia (Tang et al., 2007; Lehnardt et al., 2007). TLR2 knockout mice displayed less damage size (Tang et al., 2007; Lehnardt

et al., 2007) and neurological deficits (Tang et al., 2007) compared to wild-type mice after a focal cerebral ischemia. Neurons from TLR2 knockout mice were protected against cell death induced by glucose deprivation (Tang et al., 2007). Furthermore, Ziegler and colleagues (2007) found TLR2 was the most significantly up-regulated TLR in the ipsilateral brain hemisphere in a mouse model of transient focal cerebral ischemia. In contrast to TLR4, there are fewer studies on TLR2 in immature brain injury. A study by Stridh et al. (2011) found mRNA expression of TLR2 was up-regulated after neonatal hypoxia-ischemia. The anthors also showed TLR2 deficient mice had less infarct volume than wild type mice. However, administration of TLR2 ligand, lipoteichoic acid, does not influence sensitization to hypoxia-ischemia in developing rats (Eklind, et al., 2004). Moreover, the severity of neonatal hypoxia-ischemia is not affected by MyD88 gene absence (Mallard et al., 2009). To summarize, in adult animals, the expression change of TLR2 corresponds to the progression and severity of ischemia, suggesting that TLR2 has a role in regulating adult ischemic stroke, but few studies show the effects of TLR2 on neonatal brain injury.

TLR3 may be important in neonatal brain injury because activation of TLR3 inhibits axonal growth and might limit CNS regeneration after injury (Cameron et al., 2007). Administration of TLR3 ligand polyI:C to pregnant rodents results in impaired subcortical dopaminergic activity and cognitive function in offspring (Ozawa et al., 2006). Together, studies suggest TLR2 and 4 have destructive effects on adult ischemic brain injury (Caso et al., 2007; Cao et al., 2007; Caso et al., 2008), and activation of TLR3 might play an adverse role in immature brain injury. Under the different conditions of adult brain injury or immature brain injury, the same TLRs play different roles. Due to

the relative difficulty in manipulating neonatal animals, fewer studies have investigated immature brain injury compared to adult brain injury.

Nuclear Factor κB in brain injury. Transcription factors are other essential components in inflammatory pathways. Transcription factors are proteins that can bind to specific DNA sequences and then activate the transcription of downstream genes. Thus, the function of a transcription factor is to regulate downstream gene expression. NF-κB is a transcription factor involved in the regulation of gene expression of inflammatory cytokines (Baeuerle & Henkel, 1994). NF-κB has also been implicated in processes of synaptic plasticity (e.g. Albensi & Mattson, 2000) and memory (e.g. Freudenthal et al., 1998). NF-κB is normally located in the cytoplasm and bound to its endogenous inhibitor protein IκB. NF-κB can be activated only after it dissociates with IκB. An activated upstream IκB kinase can phosphorylate IκB to dissociate with the complex of NF-κB and IκB. The free NF- κB translocates to the nucleus, binds to κB sites, and then activates downstream genes transcription (Baeuerle & Henkel, 1994). Many genes related to inflammation contain functional κB sites, including tumor necrosis factor-α (TNF-α), intercellular adhesion molecule-1 (ICAM-1), cyclooxygenase-2 (COX-2), iNOS and interleukin-6 (IL-6) (Wang et al., 2007). Transcription of the genes related to inflammation may be regulated by NF-κB during and following brain injury.

The function of NF-κB in neonatal and adult brain injury has been investigated in many studies to identify how it behaves as a mechanism in the post injury process (e.g. Nijboer et al., 2008a; Nijboer et al., 2008b; van den Tweel et al., 2006; Schneider et al., 1999; Hill et al., 2001). NF-κB, a transcription factor, plays both beneficial and detrimental roles in neonatal hypoxia-ischemia (Nijboer et al., 2008a; Nijboer et al.,

2008b).Inhibition of NF-κB early after neonatal hypoxia-ischemia (0.5-6 hours) can result in reduced damage volume 6 weeks after injury (Nijboer et al., 2008a). In contrast, inhibition of NF-κB at both early and late time (0-12 hours) of neonatal hypoxia-ischemia leads to aggravated damage after injury (Nijboer et al., 2008b; van den Tweel et al., 2006). In neonatal hypoxia-ischemia, the timing of NF-κB activation may play a critical role in the ultimate outcome of injury, destruction or protection.

NF-κB has destructive and protective roles, in adult cerebral ischemia (Hill et al., 2001). Mice lacking one of the NF-κB subunits p50 displayed reduced ischemic damage (Schneider et al., 1999). In contrast, rats given a NF-κB inhibitor (diethyldithiocarbamate) exhibited enhanced neuronal fragmentation and increased infarct size compared to control rats in a transient middle cerebral artery occlusion (MCAO) model of ischemia, indicating a beneficial role of NF-κB (Hill et al., 2001). In adult ischemic stroke, the role of NF-κB may be more associated with the severity of the stroke rather than the timing of activation of NF-κB. Mild stresses with low transient levels of NF-κB activation may be protective, but intensive insults with high level of NF-κB may be toxic (reviewed in Vexler & Yenari, 2009). NF-κB also plays a dual role in adult cerebral stroke, which may be related to the degree of brain injury.

In summary, NF-κB is an important transcription factor in inflammatory pathways. Following brain injury, NF-κB can initiate the gene expression related to inflammation and regulate the process of brain injury. NF-κB has a dual role in both neonatal HI and adult ischemic cerebral stroke. In neonatal brain injury, the function of NF-κB may be associated with the timing of activation. In adult brain injury, its function may be more related to the severity of injury.

Cytokines in brain injury. The production of cytokines is one of the end points of innate immunity. Of these cytokines, IL-1 and TNFα are the two most studied cytokines in both developing and mature brain injury. A number of studies suggest that IL-1 can potentiate brain injury. In the adult rodent model of focal cerebral ischemia, IL-1β administration increases brain injury (Yamasaki et al., 1995), whereas mice lacking both IL-1 isoforms (IL-1α and IL-1β) have decreases of infarct size compared to wild type mice (Boutin et al., 2001). Administration or overexpression of IL-1 receptor antagonist (IL-1ra) has been shown to reduce neurologic deficits and infarct size in adult rodents following focal ischemic stroke (Allan et al., 2005; Relton et al., 1996; Betz et al., 1995). IL-1 receptor has two isoforms, type I receptor (IL-1R1) and type II receptor (IL-1R2), but only IL-1R1 is thought to account for transmitting the inflammatory effects of IL-1 (Rothwell and Luheshi, 2000). However, it is still unclear whether IL-1 plays a role in inflammation exclusively through IL-1 receptor type I. One study shows that the IL-1R1 knockout mouse has protective effects following focal ischemia (Lazovic et al., 2005), but another study of ischemic brain injury did not show these effects (Touzani et al., 2002). These two different studies of IL-1R1 suggest IL-1 may also have other IL-1R1-independent signaling pathways.

In neonatal hypoxia-ischemia, the expression of IL-1β is increased (Hedtjarn et al., 2004; Hagberg et al., 1996). The extent of inflammation and brain injury trigged by IL-1β is greatly dependent on age (Anthony et al., 1997). Anthony et al. (1997) found juvenile rats were more susceptible to IL-1 β injected into the brain compared to neonatal rats. Genetic deletion of IL-1α or IL-1β alone, or in combination, does not show reduction in brain injury in PND 9 mice following hypoxia-ischemia (Hedtjarn et al.,

2005). This study supports that the function of cytokines depends on age in immature brain. The action of IL-1 can be neutralized by its antagonist IL-1ra. The imbalance between IL-1 and IL-1ra (increase of IL-1 concomitant with decrease of IL-1ra) might play a role in the initiation of perinatal brain damage (Girard et al., 2008). The action of IL-1 in developing brains is different from adult brains. Age is an important factor that should be taken into account. IL-1 may be functional at a specific time during neonatal brain injury. The regulatory mechanisms of IL-1 including IL-1R and IL-1ra might be also altered following neonatal brain injury in order to accurately regulate the production and action of IL-1.

TNFα has effects on brain injury. TNFα exhibits pleiotropic functions, which have both destructive and protective effects on the mature ischemic brain due to the activation of different TNFRs (TNFR1 or TNFR2) (Liu et al., 1994). Inhibition of TNFα reduced ischemic brain injury and the administration of TNFα increased brain damage after ischemia (Feuerstein et al., 1998; Barone et l., 1997), indicating TNFα plays a destructive role in cerebral ischemia. In contrast, mice deficient in TNF receptor (TNFR) displays larger damage size compared to wild type mice after cerebral ischemic stroke (Bruce et al., 1996), suggesting TNFα serves a neuroprotective function. One reason for these opposing functions might be due to different TNFRs activated during TNFα signaling. Two different kinds of TNFR have been detected to date. TNFR1 contains a death domain, which mediates cell death or cell survive, and a Fas-associated domain, which is related to apoptosis (reviewed in Vexler & Yenari, 2009). In contrast to TNFR1, TNFR2 has anti-inflammatory and anti-apoptotic functions (Lovering & Zhang, 2005).

The activation of different TNFRs may result in different outcomes in adult brain injury. Unfortunately, the role of TNFα in neonatal hypoxia-ischemia is still unclear.

TNFα expression is up-regulated in rats after neonatal hypoxia-ischemia (Wang et al., 2013) and deletion of TNF gene cluster decreases LPS-mediated sensitization of the neonatal brain to hypoxic-ischemic injury (Kendall et al., 2011). Neonatal hypoxia-ischemia also induces the up-regulation of Fas death receptor (Graham et al., 2004). Fas death is one of the domains (Fas-associated domain) in TNFR1. Neonatal mice lacking functional Fas death receptor are resistant to hypoxia-ischemia brain injury compared to wild type mice (Graham et al., 2004). This study suggests TNFR1 is expressed in neonatal brain and plays a role in brain injury after hypoxia-ischemia. The neuroprotective effect of TNFα is unclear in neonatal brain injury. Whether TNFα has a time-dependent effect has also not been determined.

Immune responses: destructive or beneficial. The concept that immune responses lead to neurodegeneration, or detrimental outcomes, has dominated the literature for a long time. However, recent data show immune responses may also have neuroprotective function after brain injury (reviewed in McCombe & Read, 2008). Microglia cells are the resident immune cells in brain and produce a set of pro-inflammatory cytokines. These cytokines, one the one hand, can contribute to the progression of tissue damage, on the other hand, they are associated with the removal debris, growth and repair processes, and enhanced functional recovery (Stoll et al., 1998; Gehrmann et al., 1995; Schwartz et al., 2006). Moreover, anti-inflammatory cytokines are also produced by microglial cells, e.g. IL-10. In addition, microglia cells may produce neurotrophins, such as BDNF and IGF-1, which can benefit neural recovery (Madinier et

al., 2009). In adult brain injury, ischemia stroke can give rise to cell proliferation in subventricular zone (Parent et al., 2002), in which inflammation may play a positive role.

There are more complexities of immune responses in the fetus and infants compared to adults since the immune system of fetus and infants do not fully resemble that of an adult, and fetus and infants have a developing brain (reviewed in Rezaie & Dean, 2002). Thus, the age, as an important factor, may be taken into account in the injury in the developing brain. Increased neurogenesis has been found in juvenile (PND 21) mice brain than immature (PND 9) mice brain after hypoxia-ischemia (Qiu et al., 2007), indicating the neurogenesis is related to the age of occurrence of hypoxia-ischemia. At different age, immune responses may play different roles in brain injury during developing period.

In the processes of brain injury, the role of inflammation in brain injury, destructive or protective, may depend on the severity of brain injury and the time progression of the brain injury (reviewed in Rezaie & Dean, 2002, Vexler & Yenari, 2009). Inflammation will have more destructive effects if the brain injury is severe. Immune responses may be toxic at the beginning of the brain injury, but may be beneficial during the repair processes. It is important to understand the time points when the inflammation turns from destructive to protective. A deeper understanding of immune responses in brain injury is essential for the development of strategies for neuroprotection.

Hypoxia is a harmful event during pregnancy. In the pathophysiological process of hypoxia, stress hormones and immune responses play important roles. Excess

glucocorticoids impair neural cells and brain structure. Immune responses may contribute to secondary brain insult and may be also related to recovery mechanisms after hypoxia exposure. Hypoxia occur in the early third trimester may influence brain remodeling and synapse formation and may result in developmental disability. How motor behavior develops and stress hormones and immune system respond to hypoxia in the early trimester remain less clear. It is important to investigate the effects of hypoxia on stress hormones, immune system, and spontaneous motor behavioral development.

A model of perinatal hypoxia in rats

Fetal development is different between humans and rats, but there are comparisons that can be made. In contrast to the 9-month gestation of the human, the rat only has 3 weeks of gestation. At postnatal day (PND) 10-14, the maturation of rat neocortex is comparable to the maturation of human neocortex within the gestational period of 37-42 weeks (Romijn et al., 1991). The third trimester in humans is approximately equivalent to PND 0-12 in rats (Puglia & Valenzuela, 2010). The specific perinatal period in rats of interest, 2 days before delivery to 3 days after birth, was approximately comparable to the gestational period of 25-31 weeks in humans, a time when extremely and/or very preterm birth occurs (World Health Organization (WHO), 2013; Costeloe et al., 2012; Bonet et al., 2013). A prospective national cohort study showed the survival rate was only 2% for the infants born at 22 week's gestation, but significantly increased to 77% at 26 weeks in 2006 in England (Costeloe et al., 2012). For infants born around 32 weeks, 100% survived in 10 European regions in 2003 (Arpino et al., 2010). Based on the above literature, the survival rate of extremely preterm birth is positively dependent on the gestational age. This trend suggests a critical period during

gestation, specifically from weeks 25-31, in which only some of the infants survive premature birth. Better understanding of the pathophysiological process during the perinatal period in rats will allow researchers to better understand the mechanisms of extremely or very preterm birth in humans. The present study of hypoxia 2 days before delivery and 3 days after birth in rats is thought to reduce the mortality rate of extremely preterm infants.

A good model of perinatal hypoxia in rats should mimic most of the characteristics of clinical cases associated with preterm birth with brain injury. The animal model should alter the morphology of the brain, which may occur in preterm birth. Schwartz et al. (2004) found that rats exposed to a chronic postnatal hypoxia, 9.5% oxygen level for 30 days, had a 25% reduction in total cortical cell numbers. They also found glia cells were reduced by 34% and 41% after 10 and 30 days of hypoxia, respectively, whereas neuronal numbers were only significantly reduced by 14% after 30 days of hypoxia. There is a lack of literature examining physiological characteristics of the hypoxic models in mammals. In the chick embryo, different levels of hypoxia (5, 10, and 15% oxygen) occurred in different incubation ages, causing a depression of heart rate and blood pressure (Crossley et al., 2003). Based on the above literature, hypoxia (10 ± 1% oxygen) may result in morphological and physiological changes. Ment et al. (1998) found the level of hypoxia (9.5% oxygen) compared to that of preterm infants with bronchopulmonary dysplasia and ventriculomegaly. A rat model of perinatal hypoxia, 10% oxygen level during 2 days before delivery and 3 days after birth, was used in a study in Dr. Ivanco's lab and the morphological measurement of neurons in motor cortex was quantified (Hartle & Ivanco unpublished data, 2010). The authors found a decrease

in dendritic length, complexity and volume in layer II neurons. Based on the two studies (Ment et al., 1998; Hartle & Ivanco unpublished data, 2010), the 10% ± 1% oxygen level caused morphological changes of the brain, which may reflect some of the features of preterm birth. The study of extremely preterm birth is cirtical because the knowledge gained is thought to make more preterm infants have a better chance for survival and reduce the associated developmental disability that occurs much later.

Chapter 2

Effects of Perinatal Hypoxia on Spontaneous Motor Behaviors

In humans, many factors can cause perinatal hypoxia, such as drug abuse, anemia, pulmonary disease, umbilical cord occlusion, and uterine artery narrowing (Zhang, 2005; Rees et al., 2011). Hypoxia may result in reduced oxygen delivery to the developing brain during perinatal period. There are numerous harmful downstream effects (e.g. extra secretion of stress hormones and activation of immune system) secondary to perinatal hypoxia, which are likely to play a major role in brain injury. It is unclear whether normal developmental milestones are delayed after brain injury induced by perinatal hypoxia. Clarification of behavioral development after perinatal hypoxia is a necessary contribution to evaluation of brain injury.

Studies have shown a chronic fetal deficiency of oxygen in arterial blood results in reduction in brain weight, delayed axonal myelination, and reduced cell numbers in the central nervous system (Rees et al., 2011; Mallard et al., 1999). Mallard et al. (1999) found animals that were exposed to chronic fetal hypoxemia have enlargement of the lateral ventricles most likely resulting from reduced growth of neural processes and reduced neuronal numbers in cross-sectional area of the cerebral cortex, striatum, and hippocampus. Hartle & Ivanco (2010) investigated the morphological change of neurons in motor cortex after perinatal hypoxia and found a decrease in length, complexity and volume in layer II neurons. The excitatory amino acid, glutamate, which is released during the pathophysiologic process of hypoxia-ischemia, can disrupt the processes of normal synaptic plasticity (Johnston et al., 2009). Sensory and motor experience is one of the important factors that affect the synaptic organization of the brain (reviewed in Kolb

& Teskey, 2012). After brain injury, sensory and motor function is reduced, the stimulation of synaptic plasticity may be also reduced, and eventually synapse formation is likely decreased. Together, all the pathophysiologic process in brain may result in behavioral deficits, brain developmental delay, and impaired cognition in the later life.

A set of spontaneous behavioral tests are usually used to evaluate developmental progression or retardation between control and experimental rats. Hartle and Ivanco (2010) examined the effects of perinatal hypoxia on spontaneous behaviors and they found mild deficits. In our study, we focused on motor behavioral changes after perinatal hypoxia and assessed a variety of spontaneous behavioral tests in order to give a more complete profile of development of spontaneous abilities after perinatal hypoxia. Additionally, behavioral tests were an indicator used to evaluate the effects of stress hormones and immune responses on brain injury after perinatal hypoxia. It was hypothesized animals exposed to perinatal hypoxia would exhibit mild deficits in spontaneous motor abilities.

Subjects

Long Evans rats were housed in a standard colony room on a 12-hour light/dark cycle and had access to food and water *ad libitum*. Ethics approval was obtained through the Fort Garry Campus Animal Care Committee at the University of Manitoba. Eight pairs of adult rats were mated. Vaginal swabs were done daily, and the presence of sperm was taken as evidence of mating. This day was counted as Embryonic Day 0 (E0). The weight of the rats was monitored after mating. The increase of weight from E0 to E20 more than 100 grams indicated the rats were pregnant. In total, sixty two pups were

used in our study. Animals were selected randomly from the litters. There were 22 pups were used in the behavioral tests, 9 control animals and 13 experimental animals for behavioral assessment of spontaneous motor activities. One hypoxic pup died at PND 4 and was replaced by another hypoxic pup in the righting reflex test.

Hypoxia Induction

On E20 (approximately two days before delivery) four of the pregnant females were placed in a hypoxic chamber with 10% oxygen delivery. The chamber was acrylic and wood (approximately 20 x 24 x 24 inches), which could accommodate two standard laboratory shoebox cages. Two sides were acrylic and allowed substantial light into the box. The box housed an extremely sensitive oxygen sensor (Alpha Omega Instruments) and valve arrangement to allow the mix of nitrogen and breathing air into the chamber. The oxygen levels were achieved by regulating the mixture of nitrogen and breathing air obtained from gas cylinders. After delivery, dams and pups were kept in the hypoxic chamber for another 3 days. The 4 control dams were placed in the normoxic condition and the oxygen level was in the range of 21% ± 1%. These control and hypoxic animals were used in behavioral measures.

Behavioural Measures

All animals were tested on measures related to untrained behaviours. These behavioral measures are rat's spontaneous activities, which also mean no skill learning was expected to have taken place by the animals. The untested pups waited for testing in another room next to the testing room.

Ambulating behavior. Ambulation activity through the use of forelimbs and upper torso was assessed on PND 4, PND 8, PND 10, and PND 15 (as per Alles et al., 2010). Each pup was observed for 5 minutes on a wooden surface (approximately 50cm x 50cm). Triplicate trials were done per testing day. The movements were categorized as follows: No movement= 0, slow but impaired (e.g. asymmetric) movement= 1, slow movement= 2, and fast movement= 3.

Righting behavior. Righting was tested on PND 3, PND 4, PND 6, PND 8, and PND 10 (as per Garey et al., 2005). The righting response was tested by gently placing the rats onto their backs on a cotton sheet. A stopwatch was used to quantify the time required to return to prone position. The duration of the test was limited to 2 minutes and was conducted three times per testing day. Sessions were videotaped on each day and the tapes were analyzed for the side (right or left) that animals roll onto the quadruped position.

Negative geotaxis. Negative geotaxis was tested at PND 7, PND 11, and PND 15 (as per Alles et al., 2010). Negative geotaxis reflects the pups' latency to turn 180° to an upright position on a sloped surface. Each pup was placed head downward on an inclined plane of 50 cm x 10 cm with 25° slope. Then the pup was held gently on the starting position for 5 seconds before it is released. The time required, and direction of movement taken to face incline position was recorded. The maximum time allowed to turn upward was limited to 3 minutes. Each rat was tested three times on each testing day.

Explorative behavior. Explorative behavior was tested at PND 11, PND 16, and PND 21 (to not overlap with other tests) (as per Garey et al., 2005). Animals were placed

in the center of an open wooden enclosure and allowed to explore freely for 20 minutes. The open wooden enclosure was 60 cm long, 60 cm wide and 50 cm high. An open source tracking software "Option Control" was used to track the exploration of rats. A camera connected to the computer was mounted above the wooden apparatus. The tracking information obtained from the camera was transferred into coordinate numbers by the software and recorded in a notepad file in the computer. The total distance traveled was quantified by importing data into an Excel spreadsheet to calculate the absolute values between two pixel values. The summarized absolute values were the total distances traveled and then total distances were converted into centimeters.

Strength test. Forelimb suspension was tested at PND 15, PND20, PND 30, PND 40, and PND 50 (as per Garey et al., 2005). To assess forelimb strength, rats were allowed to grasp a wooden dowel (with a soft pillow placed underneath) with their forepaws. The apparatus was set up to accommodate the animal as it grew. There were three dowels of different thickness and height. Younger animals (PND 15 and 20) were placed on the lowest and thinnest dowel, PND 30 animals were placed on the next thickest and highest dowel, and the oldest animals (PND 40 and 50) were placed on the highest and thickest dowel. After the animal held on the dowel and was released by the experimenter, timing commenced with a stopwatch. The falling time was recorded and the maximum time allotted was 2 minutes. The animals were tested in triplicate on each testing day.

Cylinder exploration. Cylinder exploration behavior was tested at PND 10, PND15, PND 25, and PND 35 (as per Alles et al., 2010). A transparent methacrylate cylinder 20 cm in diameter and 30 cm in height was on a glass surface with a mirror

positioned beneath at a 45 degree angle. A camera was aimed at the mirror and the wall of the cylinder was seen symmetrically so that all the movements of the rat were observed. To assess forepaw preference, rats were placed in the transparent cylinder for 5 minutes. In this situation, the natural response was to search for an escape route. The number of times a rat rears as well as the paw initially used to support its body against the cylinder, were videotaped. A rear is defined as an ascent in which the rat raised its forequarters in a vertical direction and concurrently contacted the cylinder wall with one or both forepaws. The total number of rearing and the paw (right, left or both) it initially used to support its body against the cylinder was recorded.

Ladder runway. The ladder runway task was tested on PND 60 (as per Derksen et al., 2007). The regular pattern of rungs on the ladder was the only task from the paradigm used in this study. Animals were pre-trained for three days on a flat runway beginning on PND 57. The purpose of pre-training was for the rats to learn that treat pellets were located in a goal box at the end of the runway. After the pre-training sessions, animals traversed a half runway that was basically one ladder that had been laid flat. The ladder made from wood was 91cm long and 9 cm wide. The wooden rungs (8 mm diameter) were spaced at 2 cm intervals. Each animal was tested 10 times on the testing day. The time was recorded with a stopwatch. Timing commenced when the rat's front paws made contact with the runway and ended once its hind legs left the runway.

Brain weight. After decapitation brains were rapidly removed. Half of the brain was used to measure brain weight and put in 10% formalin. The other half of the brain was used to do a gene expression study and stored at -80 °C until analysis. The brains

from 18 control pups (n = 9 on PND 3 and n = 9 on PND 8) and 22 hypoxic pups (n = 12 on PND 3 and n = 10 on PND 8) were assessed.

Sample sizes. Based on previous power analysis (Hartle & Ivanco unpublished data, 2010) in Dr. Ivanco's lab, seven or greater animals per group in a behavioral study is sufficient for power. Dr. Ivanco's lab used the data from the literature (e.g. Kolb et al., 2000; Snow et al., 2008) to look at effect size and they obtained an effect. A new power analysis was not redone here.

Statistical Analysis

Each behavior was used as a dependent measure in the analysis. Sex, as a factor that may influence an animal's spontaneous activities, was assessed in the behavioral data. The data from multiple trials on one day in some tests, such as righting behavior, negative geotaxis, strength test, and ambulating behavior, were averaged before further statistical analysis was completed. The time required to return to prone position in righting behavior, the time required to face incline position in negative geotaxis, total distance traveled in explorative behavior, falling time in strength test, and the paw preference (left, right, or both) and the total number of rearings in cylinder exploration were analyzed by repeated measures 3-way ANOVAs (condition x sex x days), with days as the repeated measure. Time required to traverse one ladder in ladder runway was analyzed by repeated measures 3-way ANOVA (condition x sex x trials) with trials as the repeated measure. Brain weight was analyzed by one-way ANOVA. Fisher's LSD post hocs were used to do multiple comparisons if the ANOVA was significant.

Data of ambulating behavior were ordinal. The side that animals rolled into the quadruped position in righting behavior and direction of movement taken to face incline position in negative geotaxis were dichotomous data. The non-normal distributed data were analyzed by the non-parametric test, a Mann–Whitney *U* test.

The significant value was set at $p \leq 0.05$. Data are presented as mean ± standard error of the mean (SEM). All statistical analysis was performed using Statistica Visual Basic Primer Software (StatSoft, USA).

Results

Ambulating behavior. Ambulating scores in animals exposed to hypoxia were significantly lower than control animals on PND 4 and PND 8 ($U = 27.5$, $Z = 2.07$, $p < 0.05$ on PND 4, $U = 22$, $Z = 2.43$, and $p < 0.05$ on PND 8, Fig. 1). There were no significant differences of ambulating scores between hypoxic and control animals on PND 10 and PND 15 ($U = 58.5$, $Z = 0.00$, $p = 1.00$ on PND 10, $U = 52.0$, $Z = -0.43$, $p > 0.05$ on PND 15).

Righting behavior. The results of time required to return to prone position revealed that there was no main effect of condition ($F(1, 18) = 2.0$, $p > 0.05$). There was no main effect of sex ($F(1, 18) = 0.054$, $p > 0.05$). There was a main effect of days ($F(4, 72) = 17.97$, $p < 0.05$), indicating there was a decrease in time to return to the quadruped position across days regardless of experimental condition (Fig. 2A). There was no condition x day interaction ($F(4, 72) = 0.34$, $p > 0.05$). There was also no condition x sex interaction ($F(1, 18) = 0.19$, $p > 0.05$). Results of the direction (right or left) that animals rolled into the quadruped position from a Mann-Whitney *U* test showed no significant

differences between hypoxic and control groups on PND 3, PND 4, PND 6, and PND 8 ($U = 53$, $p > 0.05$ on PND 3, $U = 42.5$, $p > 0.05$ on PND 4, $U = 55$, $p > 0.05$ on PND 6, $U = 57.5$, $p > 0.05$ on PND 8 Fig 2B). However, there was a significant difference of direction between hypoxic and control group on PND 10 ($U = 22$, $p < 0.05$). The rat was at quadruped position and its head was forward, under this condition, the right side was defined as right and vice versa. If the rat's right side paws touched the tissue on the table first, it was defined as right side and vice versa. The significant difference of direction between different groups on PND 10 indicated more right directions were preferred to get to the quadruped position from supine position in the control group compared to the hypoxic group.

Negative geotaxis. Analysis of time required to face the incline position revealed no main effect of condition ($F (1, 18) = 1.33$, $p > 0.05$). There was no main effect of sex ($F (1, 18) = 1.83$, $p > 0.05$). There was a main effect of days ($F (2, 36) = 77.54$, $p < 0.01$), indicating a decrease in the amount of time animals were able to face incline position across days (Fig. 3A). There was no days x condition interaction ($F (2, 36) = 0.88$, $p > 0.05$). There was also no condition x sex interaction ($F (1, 18) = 1.63$, $p > 0.05$). Analysis of direction of movement taken to face incline position (Fig. 3B) showed no significant difference between groups at all time-points ($U = 53$, $p > 0.05$ on PND 7, $U = 49$, $p > 0.05$ on PND 11, and $U = 57$, $p > 0.05$ on PND 15).

Explorative behavior. Analysis of total distance traveled showed no main effect of condition ($F (1, 18) = 0.29$, $p > 0.05$). There was no main effect of sex ($F (1, 18) = 0.00$, $p > 0.05$). There was a main effect of days ($F (2, 36) = 28.84$, $p < 0.05$), indicating a shorter total traveling distance at PND 11 and a longer total traveling distance at PND 21.

There was no condition x sex interaction ($F (1, 18) = 1.12$, $p > 0.05$). There was a significant days x condition interaction effect ($F (2, 36) = 3.39$, $p < 0.05$ (Fig. 4). Post hoc analysis showed p value was equal to 0.064 on PND 11 and 0.059 on PND 21, indicating hypoxic animals showed a trend towards significance to travel a shorter distance during a 20-minute period than control animals on PND 11, but they had a trend towards significance to travel a longer distance than control animals on PND 21.

Strength test. There was no main effect of condition ($F (1, 18) = 0.03$, $p > 0.05$). There was a main effect of days ($F (4, 72) = 7.54$, $p < 0.05$), indicating alterations in amount of time animals were able to stay suspended across days. There was no main effect of sex ($F (1, 18) = 0.04$, $p > 0.05$). There was a significant interaction effect of days x condition ($F (4, 72) = 3.70$, $p < 0.05$). Post hoc analysis showed hypoxic animals grasped the dowel for less time than control animals on PND 15, whereas, hypoxic animals grasped the dowel for longer than control animals on PND 20 (Fig. 5). There was no condition x sex interation ($F (1, 18) = 0.44$, $p > 0.05$).

Cylinder exploration. Analysis of use of left forepaws showed no main effect of condition ($F (1, 18) = 1.11$, $p > 0.05$), no main effect of days ($F (3, 54) = 1.27$, $p > 0.05$), no main effect of sex ($F (1, 18) = 2.60$, $p > 0.05$), no interaction effect of sex x condition ($F (1, 18) = 0.03$, $p > 0.05$), and no interaction effect of days x condition ($F (3, 54) = 0.13$, $p > 0.05$) (Fig, 6A). Analysis of use of right forepaws showed no main effect of condition ($F (1, 18) = 1.26$, $p > 0.05$), no main effect of days ($F (3, 54) = 2.15$, $p > 0.05$), no main effect of sex ($F (1, 18) = 0.56$, $p > 0.05$), no interaction effect of sex x condition ($F (1, 18) = 0.08$, $p > 0.05$), and no interaction effect of condition x days ($F (3, 54) = 0.17$ $p > 0.05$) (Fig. 6B). Results of usage of both forepaws showed no main effect of

condition ($F (1, 18) = 2.21$, $p > 0.05$), no main effect of sex ($F (1, 18) = 0.91$, $p > 0.05$), no interaction effect of condition x sex ($F (1,18) = 0.05$, $p > 0.05$), and no interaction effect of days x condition ($F (3, 54) = 1.48$, $p > 0.05$). There was, however, a significant main effect of days ($F (3, 54) = 43.03$, $p < 0.05$), indicating an increase of usage of both forepaws to explore across days regardless of conditions (Fig. 6C). Analysis of total number of rearings showed a main effect of condition ($F (1, 18) = 4.41$, $p < 0.05$), indicating the total number of rearings is different between two groups. There was also a main effect of days ($F (3, 54) = 36.56$, $p < 0.05$), indicating an increase of numbers of rearing across days, but there was no interaction effect of days x condition ($F (3, 54) = 1.17$, $p > 0.05$) (Fig. 6D). Post hocs revealed that on PND 25 ($p < 0.05$) hypoxic rats did less rearing than control rats. This difference was not present on the other three time-points, PND 10, PND 15, and PND 35. There was no main effect of sex ($F (1, 18) = 0.67$, $p > 0.05$) and no interaction effect of condition x sex ($F (1, 18) = 0.14$, $p > 0.05$).

Ladder runway. Results showed there was no main effect of condition ($F (1, 18) = 0.05$, $p > 0.05$). These results indicated no significant difference in the time to traverse a ladder runway for the hypoxic group and control group (Fig. 7). There was no main effect of sex ($F (1, 18) = 0.55$, $p > 0.05$) and no interaction effect of sex x condition ($F (1, 18) = 0.72$, $p > 0.05$).

Brain weight. Analysis of brain weight showed no main effect of condition ($F (1, 31) = 0.05$, $p > 0.05$). There was a main effect of days ($F (1, 31) = 1599.87$, $p < 0.05$), indicating brain weight increased over time (Fig. 8). There was no main effect of sex ($F (1, 31) = 2.45$, $p > 0.05$). There was no condition x days interaction ($F (1, 31) = 0.13$, $p > 0.05$) and no condition x sex interaction ($F (1, 31) = 1.65$, $p > 0.05$).

Discussion

No gross deficits in the behavioral tests were obvious in hypoxic animals, but some differences from control were observed in our more sensitive tests. Analysis of ambulation showed the hypoxic animals had delayed development of ambulating behavior, as the scores of hypoxic animals on PND 4 and PND 8 were lower relative to control animals. Generally, the development of quadruped locomotion in rats is that spontaneous forelimb movements can be observed by day 4 and 5, raising the head and crawling appear by day 8, the beginning of quadruped walking is at days 10 and 11, and the predominant style of locomotion starts at days 12 and 13 (Altman & Sudarshan, 1975). In our study, the hypoxic animals had delayed appearance of forelimb movements and crawling compared to control animals. The motor cortex involved in locomotion activity may be impaired after perinatal hypoxia. A previous study has shown hypoxia exposure results in a decrease in length, complexity and volume in layer II neurons in motor cortex (Hartle & Ivanco unpublished data, 2010), which may be the structural and cellular foundation of delayed ambulation in hypoxic animals. At the late stage of ambulation development, PND 10 and PND15, no delayed ambulating behavior was found in the hypoxic group. Thus, the delayed ambulation was only found at the early stage of development. Human studies also showed motor delay was found in early childhood of extremely preterm infants (Doyle & Anderson, 2010) and the infants who experienced traumatic brain injury had significantly poorer gross motor performance at an earlier age (18 months), but not at later ages (30 and 42 months) (Pomerleau et al., 2012). The delayed ambulating behavior in this present study is consistent with these human studies of preterm and traumatic brain injury, indicating hypoxia exposure, as

other brain injuries, may delay motor development. The normal ambulating behavior at the later time in the hypoxic group suggests, as the animals matured, they may catch up with the normal ambulation development.

Analysis of the direction of rolling in righting behavior showed the control animals used their right sides more to roll onto the quadruped position on PND 10, but not on PND 3, PND 4, PND 6, and PND 8, but the hypoxic animals did not show any side preference at all four time points. Usually, the speed of righting on a surface increases with age, which is found in alternative studies using this test as well as our study (Altman & Sudarshan, 1975; Khalki et al., 2012; Woodworth et al., 2011). No other study analyzed the direction of rolling, meaning the direction in righting behavior may not be as important as the speed. We also need to consider, in general, rats do not show a global paw preference (Whishaw et al., 1990). The side preference of rolling may occur when motor functions are more fully developed, although the preference could be temporary. Lack of side preference in hypoxic animals indicates there may be a delayed motor development in hypoxic animals.

The cylinder test is used to test sensorimotor function. Usually, the basal ganglia, sensorimotor cortex, and related systems throughout the central nervous system are involved in the cylinder test (Schallert & Woodlee, 2005). The number of rearings within the cylinder can be used to examine the development of sensorimotor activities. Usually, the cylinder test is used to assess the unilateral rat models of central nervous system injury such as focal cerebral ischemia and unilateral spinal cord injury. The cells changed in the motor cortex after perinatal hypoxia in rats (Hartle & Ivanco unpublished data, 2010) and we assumed in our study both sides of the sensorimotor cortex are influenced

by hypoxia, such that the number of rearings may decrease and the asymmetrical use of forelimbs may not be present. The result from our study agreed with the above assumption, as analysis of total number of rearings in the cylinder test showed a significant difference between the two groups on PND 25, but no significant differences in the usage of left or right forelimbs. Rats preferred to use both forelimbs to rear regardless of conditions indicating no unilateral damage of sensorimotor cortex in hypoxic animals. Total number of rearings was significantly decreased suggesting that both sides of sensorimotor cortex may be impaired to some extent in hypoxic animals and hypoxic rats may have a more generalized sensorimotor developmental delay. The total number of rearings was not significantly different at the younger age (PND 10 and PND 15) since it was difficult for young rats to raise their body to rear. As rats grew, they were able to rear on PND 25, but hypoxic rats had decreased total number of rearing compared to control rats, indicating the hypoxic pups had sensorimotor developmental delay. At the older age of PND 35, rats were in early adolescence and no significant differences of sensorimotor activity were found between groups. It is possible that, as the hypoxic animals matured, they caught up the sensorimotor development. Rousset et al. (2013) found brain injury from maternal inflammation led to motor dysfunction in neonatal rats only at early age (before 5 weeks age), and pups reached control level by 5 weeks. Human studies showed motor delay in extremely preterm infants (Doyle and Anderson, 2010, Pomerleau et al., 2012). Thus, the result of cylinder in our study is generally consistent with other studies found in the literature and indicates a delayed sensorimotor development in the hypoxic group.

The total distance traveled in an open field box is a behavioral test to evaluate locomotor activity. Hypoxic animals had less total distance traveled in an open field test compared to control animals on PND 11 and the difference approached significance, indicating the locomotor activity may be delayed to some degree in hypoxic animals. The total distance traveled was longer in hypoxic animals than control animals on PND 21 and the data approached significance, suggesting possible hyperactivity present in hypoxic animals. Hyperactivity has been reported in several studies of brain injury in animals (Cai et al., 1999; Gerhardt & Boast, 1988; Karaswa et al., 1994). The results of open field task may indicate the delayed motor behavior in the hypoxic animals, but there is another explanation.

Bursian et al. (1994) reviewed the process of motor activity development during the first 2-3 weeks and found: an increase in the activity and, then, the subsequent inhibition of the activity with a shift of the corresponding phases. This phenomenon is related to the maturation of excitatory and inhibitory systems of regulation of the nervous activity. These findings might explain why there was a decreased distance traveled on PND 16 in our study. During the two first testing days (PND 11 and PND 16), the locomotor of rats was still developing. The inhibitory system that can lead to deep functional rearrangement in the brain might be more active around PND 16, which resulted in the less locomotor activity and less distance traveled. On PND 21, the locomotor activity was almost well developed, so rats traveled the longest distance compared to the first two testing days at the younger age. There was a distinctive pattern of total distance traveled in the open field test, a longer distance on PND 11, a shorter distance on PND 16, and the longest distance on PND 21.

Analysis of suspension time in the strength test showed significantly less suspension time in the hypoxic animals than in the control animals on PND 15. This result is generally consistent with studies in the literature, indicating suspension time on a dowel was reduced in newborn hypoxic animals (Grojean et al., 2003). This reduced suspension time indicates there may be growth retardation in hypoxic animals and, perhaps, they had muscle weakness relative to their size, explaining less suspension time. However, an opposing result was found on PND 20 in our study. The hypoxic animals had a longer suspension time on a dowel than recorded in the control animals. It is possible the hypoxic animals had a lighter body weight, leading to better performance in this test, although we did not weigh them. Pozo et al. (2012) found rats exposed to postnatal intermittent hypoxia had decreased body weight during the first 3 weeks, indicating growth restriction of the hypoxic rats. In our study, the decrease in body weight may be also in the perinatal hypoxic animals. The strength test may have high correlation with body weight regardless of whether they were in the control or experimental condition. This correlation was found in our study in the other testing days at older age, when animals grew older and heavier suspension time decreased. The suspension time in this strength test may indicate growth retardation of the hypoxic animals, especially at the early age.

Analysis from the reflex test (righting behavior and negative geotaxis) showed the time required returning to prone position and the time required to face incline position was not significantly different between the hypoxic group and the control group, but there were significant effects of days, which meant as rats grew they used less time to complete these tests. Also, analysis of the ladder runway did not show any difference between the

hypoxic group and the control group. It is possible rats, which were young adults on PND 60, were mature enough and the ambulation and motor activity had been totally developed. Analysis from these tests (righting behavior, negative geotaxis, and ladder runway) did not show significant differences between the two groups, indicating no reflex deficits in hypoxic animals and no locomotor impairment in hypoxic animals at young adult age. The analysis of brain weight did not show any significant differences between the hypoxic group and the control group at PND 3 and PND 8. Our data for brain weight are consistent with a previous study in Dr. Ivanco's lab (Peloquin, Hartle, and Ivanco unpublished data, 2010/personal communication) and with other published data, in which chronic mild prenatal hypoxia had a subtle effect on brain weight (Gross et al., 1981). Our result of brain weight indicated perinatal hypoxia in rats is not severe and may cause mild behavioral deficits. There was no main effect of sex, indicating sex did not influence the spontaneous behavioral development in both the hypoxic group and control group.

Many studies have focused on behavioral evaluation after brain injury. Some studies did not find significant behavioral impairment (e.g. Garey et al., 2005; Cada et al., 2000) including the similar study of frontal cortex injury on embryonic day 18 (Kolb, 1998), but other studies showed behavioral deficits (e.g. Karalis et al, 2011; Khalki et al., 2012; Chehimi et al., 2012; Grojean et al., 2003). In our study, moderate deficits in behaviors may be due to the fact that the damage was mild or that spontaneous motor behaviors are hardwired in the brain and are not easily disturbed. It is also possible that the behavioral problems may have been subtle and these tasks used were not sensitive enough to detect them.

The moderate deficits in some spontaneous motor behaviors and no deficit in other motor behaviors (righting behavior, negative geotaxis and open field activity) were expected and they are generally consistent with some previous studies after mild brain injury (e.g. Garey et al., 2005; Cada et al., 2000). Garey et al. (2005) found the rats taken low dose of acrylamide (1mg/kg/day), a known neurotoxicant, did not show behavioral delay in righting reflex and open field measures, but the rats taken high dose of acrylamide (10/mg/kg/day) displayed behavioral impairment in negative geotaxis performance. Postnatal treatment with alpha-difluoromethylornithine (DFMO), a potent inhibitor of ornithine decarboxylase, did not affect some behavioral measures (righting reflex, negative geotaxis and open field activity) in rats (Cada et al., 2000). Whether or not data indicate a behavioral deficit exists may depend on the extent of brain injury, such as the lesion volume, neuronal loss, apoptosis, and self-repaired abilities. Postnatal treatment with DFMO and low dose of acrylamide may cause moderate or mild brain impairment, which may result in no deficit in some spontaneous motor behaviors, including righting relex and negative geotaxis. We could predict cognitive tasks may be impaired after perinatal hypoxia based on some human and animal studies that have shown cognitive deficits well after perinatal brain injury (Lun et al., 1990; Doyle & Anderson, 2010). In the future study of perinatal brain injury, cognitive evaluation should be considered, but was outside of the scope of this project.

Perinatal hypoxia may also influence neuroplasticity. In our study, we found the delayed behavior of hypoxic animals eventually caught up to control levels, although this is not what we expected. This catch-up of motor activities is generally consistent with other studies that animals which experienced perinatal/neonatal brain injury exhibit

cognitive deficits later in life (Grojean et al., 2003; Cai et al., 1999), suggesting the alteration of neuroplasticity after brain injury may affect brain functions in the long term. The behavioral changes reflect the structural changes after perinatal hypoxia. Although we did not do morphologic examinations on brain, there should be altered structure in hypoxia exposed brain. A previous study (Hartle & Ivanco unpublished data, 2010) identified a decrease in length, complexity and volume in layer II neurons, which may be the structural basis of deficits in behavioral development. Continued study is ongoing in the lab.

Although the majority of spontaneous behaviors examined in our study did not show a significant difference between hypoxic and control groups, several tasks did exhibit some mild differences between these two groups. These behavioral data demonstrate that perinatal hypoxia may result in delayed development of motor and sensorimotor behaviors and growth restriction. These behavioral deficits may reflect the extent of brain injury. The brain injury may be related to the molecular changes after perinatal hypoxia. Another finding in our study was the impaired behaviors became indistinguishable from controls with increased maturity, suggesting neuroplasticity or recovery mechanisms may contribute to this catch-up. The altered neuroplasticity may contribute to cognitive deficits later in life. Perinatal hypoxia-induced brain injury may be involved in short- and long-term behavioral impairments, but may be difficult to measure.

Chapter 3

Responses of Stress Hormones following Hypoxia

It is well recognized stress can initiate the secretion of stress hormones, such as glucocorticoids. The glucocorticoids (cortisol in humans, sheep and guinea pigs, and corticosterone in rodents) are released via the hypothalamic-pituitary-adrenal (HPA) axis. Excess glucocorticoids are detrimental to the body's vital organs, especially the brain. Perinatal hypoxia can result in excess secretion of glucocorticoids, but little is known about the expression pattern of glucocorticoids after perinatal physical and psychological stress in rats. We investigated how glucocorticoids change after perinatal hypoxia.

The general response of the brain to stress is well known. The hypothalamic paraventricular nucleus releases corticotropin-release hormone (CRH), which stimulates the production and secretion of adrenocorticotropic hormone (ACTH) into the peripheral circulation, then, ACTH initiates the synthesis and secretion of glucocorticoids from the adrenal cortex (Xiong & Zhang, 2012). Glucocorticoids act as transcription factors and are involved in the regulation of gene expression to mediate profound and diverse physiological effects on development, organization of neural cells in the nervous system, anti-inflammation, and many other functions (Blum & Maser, 2003). At physiological levels, glucocorticoids have effects on fetal development by accelerating the maturation of almost all tissues and organs, including brain (Roberts & Dalziel, 2006; Ward, 1994). Glucocorticoids play a role in normal development and maturation of the brain at the end of pregnancy, as they initiate terminal maturation, remodel axons and dendrites, modulate the progress of neural survival, and program the progress of cell death (Meyer, 1983).

The correct physiological level of glucocorticoid is important for the fetus to develop normally.

The perinatal period is considered to be a critical time for brain development because much neurological development occurs during this period. As a result, the developing mammalian nervous system is particularly vulnerable to injurious influences of stress hormones. Perinatal exposure to excessive glucocorticoids has harmful effects on neuronal structure and synapse formation (Joels et al., 2007; Matthews, 2000). Excess glucocorticoids during the perinatal period may retard the radial migration of post-mitotic neurons in the developing cerebral cortex (Fukumoto et al., 2009), induce apoptosis to result in loss of neurons (Mutsaers & Tofighi, 2012), cause significant decreases in actively proliferating neural cells, and reduce neurogenesis and increases cell death (Mandyam et al., 2008). Hence, excess glucocorticoids have numerous harmful effects on brain development.

It is well accepted that perinatal hypoxia (including prenatal and postnatal oxygen deprivation) is considered a stressor and can stimulate over-secretion of glucocorticoids, which results in the fetus and/or infant being exposed to elevated levels of endogenous glucocorticoids. Literature shows both corticosterone and cortisol can be found in rodents (Franklin et al., 2004; Jin et al., 1997). The time-course of corticosterone and cortisol fluctuation in rats after perinatal hypoxia is not well reported in the literature. We hypothesized the amount of corticosterone would increase with perinatal hypoxia. We also hypothesized levels would return to normal after return to a normoxic environment, but not immediately. We similarly hypothesized the amount of cortisol would increase after perinatal hypoxia, and, then, change over time after perinatal hypoxia.

Subjects

Long Evans rats were housed in a standard colony room on a 12-hour light/dark cycle and had access to food and water *ad libitum*. Ethics approval was obtained through the Fort Garry Animal Care Committee at the University of Manitoba. Eight pairs of adult rats were mated. Vaginal swabs were done daily and the presence of sperm was taken as evidence of mating. This day was counted as Embryonic Day 0 (E0). The weight of the rats was monitored after mating. The increase of weight from E0 to E20 more than 100 grams indicated the rats were pregnant. Animals were selected randomly from the litters. There was tissue from 18 control pups and 22 hypoxic pups used in the ELISAs.

Hypoxia Induction

On E20 (approximately two days before delivery) four of the pregnant females were placed in a hypoxic chamber with 10% oxygen delivery. The chamber was acrylic and wood (approximately 20 x 24 x 24 inches), which could accommodate two standard laboratory shoebox cages. Two sides were acrylic and allowed substantial light into the box. The box housed an extremely sensitive oxygen sensor (Alpha Omega Instruments) and valve arrangement to allow the mix of nitrogen and breathing air into the chamber. The oxygen levels were achieved by regulating the mixture of nitrogen and breathing air obtained from gas cylinders. After delivery, dams and pups were kept in the hypoxic chamber for another 3 days. The 4 control dams were placed in the normoxic condition and the oxygen level was in the range of 21% ± 1%. The control and hypoxic pups were used in evaluation of stress hormones.

Serum Collection

After exposure to hypoxia, serum was collected from 12 pups at PND 3. The dams and remaining pups were then housed at 21% ± 1% oxygen and 10 pups were sacrificed at PND 8 for serum collection. Serum was collected from eighteen pups (9 on PND 3 and 9 on PND 8) from the control group. After live decapitation whole blood was collected into a 1.5 mL microfuge tube. Blood was held at room temperature for approximately 30 minutes to allow optimal clotting and then spun for 10 minutes at 13,300 rpm. Serum was transferred into fresh microfuge tubes and the aliquots were stored at -80 °C until analysis.

Serum cortisol levels assay – ELISA

Cortisol levels were determined with the ELISA method (Cortisol Enzyme Immunoassay Kit, DetectX, USA). The supplied Assay buffer was diluted to 1:5 with deionized water, and the supplied Wash Buffer was diluted to 1:20 with deionized water. The serum was diluted with an equal volume of supplied Dissociation Reagent, then further diluted 1:50 with the supplied Assay Buffer prior running in the assay. Final serum dilutions were 1:100. Cortisol standards were prepared as per Table 1. Samples or standards (50ul) were added into wells in a plate coated with goat anti-mouse IgG. Assay buffer (75ul) was added into the non-specific binging (NSB) wells, and (50ul) was added into wells to act as maximum binding wells. The DetectX® Cortisol Conjugate (25ul) was added to each well, and then the DetectX® Cortisol Antibody was added to each well, except the NSB wells. The plate was covered and placed on a shaker at room temperature for 1 hour. The plate was washed 4 times with the wash buffer. The supplied TMB

substrate (100ul) was added to each well to develop the blue color density and incubated at room temperature for 30 minutes without shaking. The supplied Stop Solution (50ul) was added to each well and the color changed from blue to yellow. The plate was put into a plate reader (Bio-Rad, CA, USA) and the optical density (OD) of the color was read at a wavelength of 450nm. All samples were run in four replicates.

The concentrations were calculated according to the following steps. The NSB OD was subtracted from the cortisol standard and sample ODs to obtain net ODs of the cortisol standards and samples as per supplier's instructions. The concentration of the cortisol standard was logarithmicly transformed. A standard curve was created by determining the linear trendline. The sample concentration was obtained from the equation of logarithmic concentration of standard and OD. The sample concentration was obtained from the equation of net OD and concentration. The sample concentration was transformed back to conventional numbers and was multiplied by the dilution factor to obtain neat sample values, as per the manufacturer's directions.

Serum corticosterone levels assay – ELISA

Corticosterone levels were measured by ELISA using a corticosterone ELISA Kit (Abnova, CA, USA). The supplied enzyme immunoassay (EIA) Diluent was diluted to 1:10 with reagent deionized water. The supplied Biotinylated Corticosterone was diluted with 4mL EIA diluent to produce a 2-fold stock solution, and the stock solution was further diluted to 1:2 with EIA Diluent. Wash Buffer in the kit was diluted to 1:20 with deionized water. The supplied Strptavidin-Peroxidase was diluted to 1:100 with EIA Diluent. The rat serum was diluted to 1/200 with supplied EIA Diluent. Corticosterone

standards were prepared as per Table 2. The corticosterone plate was coated with a polyclonal antibody against corticosterone. Standard and/or samples (25ul) were added into the wells and immediately (25ul) of Biotinylated Corticosterone antibody was added to each well (on top of the standard or sample). The plate was covered with a sealing tape and incubated for 2 hours at room temperature. The plate was washed 5 times with (200ul) of Wash Buffer manually. When the plate was being washed, it was inverted each time and the contents decanted, and hit 4-5 times on absorbent paper towel to completely remove the liquid. Streptavidin-Peroxidase Conjugate (50ul) was added to each well and incubated for 30 minutes at room temperature. The plate was washed 5 times as described above. Chromogen Substrate (50ul) was added to each well and incubated for 20 minutes to develop blue color density. The Stop Solution (50ul) was added to each well and the color changed from blue to yellow. The plate was put into a plate reader (Bio-Rad, CA, USA) and the OD of the color was read at a wavelength of 450nm. All samples were run in duplicate.

The concentrations were calculated with the following steps. Both the concentration of the corticosterone standard and the sample ODs were logarithmic transformed as per supplier's instruction. A standard curve was created by determining the linear trendline. The sample concentration was obtained from the equation of logarithmic concentration of standard and logarithmic OD. The sample concentration was transformed back to conventional numbers and was multiplied by the dilution factor to obtain neat sample values.

Statistical Analysis

The concentration of cortisol and corticosterone from ELISA data were analyzed with repeated measures two-way ANOVA with trials as within subjects followed by Fisher's post hoc when the ANOVA was significant. The between subject variables were oxygen level (10% vs 21%) and age (PND 3 or PND 8). The significant value was set at $p \leq 0.05$. Data are presented as mean ± standard error of the mean (SEM). All statistical analysis was performed using Statistica Visual Basic Primer Software (StatSoft, USA).

Results

Serum cortisol levels. There was a significant effect of hypoxia on cortisol levels, resulting in increased cortisol concentrations, $F(1, 36) = 78.30$, $p < 0.01$. There was no significant effect of time on cortisol levels, $F(1, 36) = 0.12$, $p = 0.73$. Post hoc comparisons indicated the mean concentration of cortisol for hypoxia at PND 3 was significantly increased compared to the normoxic condition at PND 3 and at PND 8 (Fig. 9). The mean concentration of cortisol for hypoxia at PND 8 was significantly increased compared to the normoxic condition at PND 3 and at PND 8. Under the normoxic conditions, the mean levels of cortisol at PND 3 did not significantly differ from at PND 8. Similarly, under hypoxic conditions, the mean levels of cortisol at PND 3 did not significantly differ from the mean levels of cortisol at PND 8.

Serum corticosterone levels. There was a significant effect of hypoxia on corticosterone concentrations, resulting in increased levels of corticosterone, $F(1, 36) = 11.42$, $p < 0.01$. There was also a significant effect of age on corticosterone concentrations, leading to decreased levels of corticosterone with the longer time removal

of hypoxic chamber, $F(1, 36) = 9.34$, $p < 0.01$. Post hoc comparisons showed the mean concentration of corticosterone for hypoxia at PND 3 was significantly increased compared to the normoxic condition at PND 3 and at PND 8 (Fig. 10). However, the mean concentration of corticosterone for hypoxia at PND 8 was not significantly different from the normoxic condition at PND 3 and at PND 8. Under the normoxic conditions, the mean concentration of corticosterone at PND 3 did not significantly differ from that at PND 8. However, under the hypoxic conditions, the mean concentration of corticosterone at PND 3 was significantly increased when compared to the mean at PND 8.

Discussion

The concentrations of both cortisol and corticosterone were significantly increased after perinatal hypoxia in rats, supporting some of our hypotheses. Both maternal and neonatal hypoxia can stimulate the HPA axis and secrete glucocorticoids. In the present study, the concentration of corticosterone was about 10 times than the concentration of cortisol, indicating corticosterone, not cortisol, was dominant in laboratory rats, which is consistent with the literature (Velickovic et al., 2012; Xiong & Zhang,). In addition, the corticosterone levels were lower 5 days after the animals left the hypoxic chamber. The results indicate cortisol and corticosterone are increased after perinatal hypoxia, but only cortisol stays elevated. It is possible cortisol and corticosterone may have different long term actions after stress and circulations may be different in brain.

In our study, the increases of corticosterone and cortisol are possible and corticosterone and cortisol were both present after hypoxic stress. Although we did not

evaluate dam stress, it is well known that before birth the corticosterone and cortisol levels of hypoxic dams are markedly higher and cause the decrease in placental 11 β-hydroxysteroid dehydrogenase 2 (11 β-HSD2) barrier, which led to fetal overexposure of corticosterone and cortisol. This overexposure of glucocorticoids results in the high levels of basal corticosterone and cortisol and impaired feedback regulation of the HPA axis in pups after birth (de Vries et al., 2007; Levitt et al., 1996; Weinstock, 2005). After birth, the pups were in the hypoxic environment, which activated excess secretion of corticosterone and cortisol from pup's HPA axis. These increases of corticosterone and cortisol are generally consistent with studies showing excess glucocorticoids were present in the animals during prenatal stress (Weinstock, 2001) and postnatal stress (Mikhailenko, et al., 2009). In the present study, the elevated levels of corticosterone and cortisol were from the hypoxic dam and pups.

Corticosterone testing is predominant in most of the literature on laboratory rats, but studies have also shown the presence of both cortisol and corticosterone in rats (Franklin et al., 2004; Jin et al., 1997). Franklin et al. (2004) found administration of an antidepressant drug (Hypericum perforatum) reduced the levels of cortisol and corticosterone in the rat brain, but not in the serum, suggesting that both cortisol and corticosterone present in rats and they may have different pathophysiological functions in different systems. Another study of rats showed an anesthetic drug blocked the response of corticosterone following a bone-crush injury, but this drug did not change the plasma levels of cortisol (Jin et al., 1997). The study by Jin et al. (1997) indicates both cortisol and corticosterone can be identified in rats, but have different responses to an acute stressor, such as a bone-crush injury. After a bone-crush injury in rats, the corticosterone

level was increased, but no obvious alteration of cortisol. The mechanism underlying the presence of two glucocorticoids in laboratory rats is not understood at present.

It is widely assumed cortisol and corticosterone share the same physiological roles and their importance depends merely on their concentrations in serum. Although cortisol and corticosterone may act through similar mechanisms and have similar effects, increasing evidence shows they have different physiological functions (Schmidt & Soma, 2008; Schmidt et al., 2010; Vera et al., 2012; Kass et al., 1954). First, the predominant cortisol or corticosterone can be organ specific and age specific. Schmidt and Soma (2008) measured cortisol and corticosterone levels in plasma, immune organs, and brain of developing zebra finches. They found both cortisol and corticosterone were present in the plasma, but corticosterone was at higher concentrations in the plasma and increased with age. Cortisol levels were similar to corticosterone levels at birth, but decreased rapidly with age. Cortisol was at higher concentrations in immune organs and also decreased with age. In the same study, both cortisol and corticosterone levels were very low in the brain (Schmidt & Soma, 2008). The study by Schmidt and Soma (2008) explained glucocorticoids were found at different levels depending on organs and ages in zebra finches. Second, glucocorticoids may bind to different intracellular receptors, either mineralocorticoid receptors (MR) or glucocorticoid receptors (GR). MR binds endogenous glucocorticoids with high affinity and GR binds endogenous glucocorticoids with low affinity in neural cells in zebra finches (Schmidt et al., 2010). The expression of the glucocorticoids receptors may differ in different systems. For example, GR expression is less in cells of the immune system, but more in brain (Schmidt et al., 2010). Cortisol and corticosterone may function by binding to different receptors. Third, cortisol

and corticosterone have different responses to ACTH or stressors in different species (Vera et al., 2012; Kass et al., 1954). Vera et al. (2012) found in the species of *Ctenomys talarum*, a medium-sized wild rodent inhabits in a southern part of South American, cortisol had positive response to ACTH, but corticosterone did not. A study of rabbits showed corticosterone was the main stress hormone under baseline conditions, but cortisol became the main stress hormone after chronic ACTH treatment (Kass et al., 1954). Cortisol and corticosterone have different responses to ACTH, indicating cortisol and corticosterone may have different physiological functions.

In our study, corticosterone levels dropped down to baseline, but cortisol levels stayed high after hypoxic stress. Although cortisol levels were consistently high after hypoxic stress, it might not play an important role in rats because the level was relatively low compared to the baseline corticosterone levels (see figures 8 and 9). The decreased levels of corticosterone after perinatal hypoxia may be a positive response because prolonged overexposure has detrimental effects on nearly every organ and tissue in the body (reviewed in Xiong & Zhang, 2012). Our results also indicate corticosterone and cortisol may have different functions. The roles of cortisol in brain injury in laboratory rats are not very clear. More studies are required to determine the relationships between cortisol and corticosterone during and after stressful conditions. For example, future research should test more time points during and after hypoxic stress to find out the basic curve of cortisol and corticosterone expression over time.

In the present study, corticosterone had a rapid response to a stressor, but cortisol had a relatively slow response to a stressor. The literature also shows corticosterone and cortisol have different responses to stress. Studies of pubertal development following an

acute stressor indicated an acute stress-induced corticosterone secretion in pubertal rats took 40-60 minutes longer to return to baseline compared to adult rats, which took around 30 minutes to return to baseline (Vazquez & Akil 1993; Romeo et al., 2004a,b, 2006a,b). Velickovic et al. (2012) studied the time-course of corticosterone in juvenile rats after cranial irradiation and found corticosterone concentrations were raised as early as 2 hour after irradiation and returned to the normal level within 24 hours after irradiation. These studies of corticosterone levels after stress indicate corticosterone may respond to a stressor very fast, as well as return to normal levels rapidly after the animal escape a stressful environment. Most studies in rats focus on corticosterone in response to a stressor, but one study focused on the alteration of cortisol levels after spinal cord injury, which can lead to stress responses (Gezici et al., 2009). This study of cortisol in rats focused on the cortisol changes at different time points within the first 24 hours after an acute spinal cord injury (Gezici et al., 2009). Gezici et al. found cortisol levels were increased rapidly after spinal cord injury and were consistently high at 24^{th} hour, indicating cortisol had a slower response than corticosterone. It is not very clear how corticosterone and cortisol are expressed during and after perinatal stress, as few studies have explored this question.

The local 11β-HSD plays an important role in the metabolism of glucocorticoids as it can biosynthesize and inactivate steroid hormones (Blum & Master, 2003). Corticosterone and cortisol may have different pathophysiological responses to the local 11β-HSD. In our study, stress-induced different patterns of corticosterone and cortisol may be due to the different responses to local 11β-HSD. Corticorsterone may be more sensitive to local 11β-HSD than cortisol in rats, especialy after the stress of perinatal

hypoxia. Our results suggest in laboratory rats cortisol and corticosterone may respond to perinatal hypoxia, but they may have different response patterns during and after stress. Corticosterone may be a biomarker of perinatal hypoxia in rats as it had a faster response than cortisol. Further studies would be done to validate corticosterone as a biomarker. For example, the corticosterone levels under different conditions of hypoxia (e.g. different levels of oxygen and different duration of hypoxia to obtain different models represents different severity of perinatal hypoxia) would be compared to determine whether corticosterone levels are significantly different from control animals and whether they are different across different conditions.

In the present study, perinatal hypoxia, as a stressor, stimulated the excessive secretion of corticosterone and cortisol in rats. The stress responses of glucocorticoids, on one hand enhance the maintenance of critical tissue function and the capacity for survival of the organism, but on the other hand, may contribute to irreversible changes in tissue structure or function. In our study, these increased levels of glucocorticoids may result in impaired brain and behavioral development. Corticosterone had higher levels than cortisol in plasma, suggesting corticosterone may be a dominant signaling molecule in laboratory rats. Moreover, we also found corticosterone had rapid responses to perinatal hypoxia in rats. Therefore, corticosterone may be a potential biomarker of perinatal hypoxia used to predict its presence and its severity in rats.

Chapter 4

Gene Expression of Immune Responses following Hypoxia

Immune responses can be triggered by brain injury and may contribute to the secondary insult commonly reported. One of the final products of the inflammatory pathway are cytokines, which may be the main mechanisms to further insult brain tissues. The literature does not provide substantial information about the immune responses after perinatal hypoxia. Therefore, in our study, we investigated how immune responses were altered after perinatal hypoxia in rats.

Increasing evidence shows the immune system is involved in the progression of brain injury (Stridh et al., 2011; Hedtjarn et al., 2004; Kamel & Iadecola, 2012). Brain injury triggers an inflammatory cascade that progresses for days to weeks and plays a role in secondary progression of injury (Hedtjarn et al., 2004). The inflammatory cascade is characterized by activation of inflammatory cells (microglia/macrophages) that accumulate at the site of injury and release inflammatory cytokines (Bona et al., 1999, McRae et al., 1995, Zhang et al. 2010). Some animal studies have shown the detrimental effect of inflammatory cytokines such as IL-1and TNF-α on cerebral ischemic brain injury. Brain damage increased when IL-1was administered to rats in the cerebral ischemic stroke (Yamasaki et al., 1995) and IL-1 knockout mice had smaller infarcts compared to wild type (Wang et al., 2007). IL-1Rn has a function of decrease in inflammatory reactions of IL-1. Following neonatal HI in rats, IL-1Rn expression was down-regulated (Girard et al., 2008). Administration of IL-1Rn in a model of cerebral ischemia resulted in a decrease in infarct area and neuronal death (Relton & Rothwell, 1992). IL-1Rn has an anti-inflammatory effect in brain injury. The cerebral ischemic

brain damage was reduced when TNF-α was inhibited (Yang et al., 1998), whereas focal ischemic brain injury was increased when recombinant TNF-α protein was administrated after stroke (Barone et al., 1997). Clearly, immune responses, especially those of inflammatory cytokines, are involved in the process of brain injury and may be deleterious to brain injury.

Chemokines have a variety of physiological functions, such as control of migration, proliferation, differentiation and angiogenesis, and inflammation. Monocyte chemoattractant protein-1 (MCP-1) plays a role in inflammation, as well as in brain injury. In a mouse model of reversible inflammatory encephalopathy, overexpression of MCP-1 resulted in increased production of cytokines and brain injury (Huang et al., 2002). In a mouse model of focal ischemia, MCP-1 deficiency is associated with decreased infarct area and a reduction of accumulation of macrophage within infarct area (Kumai et al., 2004). MCP-1 expression has been found increased following neonatal HI in a rodent model (Xu et al., 2001). These studies suggest MCP-1 is involved in inflammation after brain injury in both neonates and adults. TLRs, pattern recognition receptors, regulate innate immune response. Studies have reported TLR2 (Tang, et al., 2007; Lehnardt et al., 2007) and TLR4 (Tang, et al., 2007; Yang et al., 2008) are up-regulated following adult cerebral ischemia in rodents. Mice deficient in TLR2 (Tang, et al., 2007; Lehnardt et al., 2007) and TLR4 (Cao et al., 2007; Caso et al., 2007; Caso et al., 2008) have less infarct area and neurological deficits compared to wild-type mice following focal cerebral ischemia. *TLRs* play an important role in adult cerebral ischemia. It appears all components in the inflammatory pathways have a potential role in the

process of brain injury. Some components may aggravate brain injury, but others may have anti-inflammatory functions and benefit brain injury.

NF-κB is a transcription factor involved in regulation of inflammation after stroke (Baeuerle & Henkel, 1994). NF-κB can induce other major genes involved in inflammation, such as TNFα, ICAM-1, COX-2, inducible nitric oxide synthases (iNOS), and IL-6. Mice deficient in NF-κB's p50 subunit are protected from cerebral ischemic brain injury (Schneider et al., 1999). NF-κB inhibition early after neonatal HI (0.5-6 h) results in neuroprotection (Nijboer et al., 2008a). NF-κB may have a dual role in brain injury.

A number of studies also found immune responses or inflammation have neuroprotective properties after brain injury, in addition to the neurotoxic properties (Woodcock & Morganti-Kossmann, 2013; Stoll et al., 2002). The literature indicates pro-inflammatory cytokines may play a beneficial neurotrophic effect after cerebral ischemia (Shalak & Perlman, 2004). Anti-inflammatory factors such as IGF-1, TGF-β1, GDNF and IL-10 are also produced by immune cells (microglia) where pro-inflammatory cytokines are released (Vexler & Yenari, 2009; McCombe & Read, 2008). Animal studies support the neuroprotective effects of immune responses on brain injury. TNF knockout mice were shown to have increased neuronal cell degeneration following ischemia and excitotoxic injury (Bruce et al., 1996). Immune responses play a beneficial role in repair after brain injury, but when this occurs is not fully understood.

Most of previous research has focused on the immune responses following cerebral ischemia in adult animals and neonatal hypoxia-ischemia, with little research

examining the perinatal period. The pathophysiological role of some immune responses (e.g. TNFα) in neonatal brain injury is unclear. Moreover, it is not clear which components in the inflammatory pathways would play a role in perinatal hypoxia. We hypothesized immune responses would be activated after perinatal hypoxia. To test our hypothesis, we quantified the gene expression of immune responses at two time points after hypoxia in order to understand the time course of immune response in brain injury after perinatal hypoxia.

Subjects

Long Evans rats were housed in a standard colony room on a 12-hour light/dark cycle and had access to food and water *ad libitum*. Ethics approval was obtained through the Fort Garry Campus Animal Care Committee at the University of Manitoba. Eight pairs of adult rats were mated. Vaginal swabs were done daily, and the presence of sperm was taken as evidence of mating. This day was counted as Embryonic Day 0 (E0). The weight of the rats was monitored after mating. The increase of weight from E0 to E20 more than 100 grams indicated the rats were pregnant and carrying good sized litters for study. Animals were selected randomly from the litters. There were 6 control pups and 6 hypoxic pups used in Real Time RT-PCR.

Hypoxia Induction

On E20 (approximately two days before delivery) four of the pregnant females were placed in a hypoxic chamber with 10% oxygen delivery. The chamber was acrylic and wood (approximately 20 x 24 x 24 inches), which could accommodate two standard laboratory shoebox cages. Two sides were acrylic and allowed substantial light into the

box. The box housed an extremely sensitive oxygen sensor (Alpha Omega Instruments) and valve arrangement to allow the mix of nitrogen and breathing air into the chamber. The oxygen levels were achieved by regulating the mixture of nitrogen and breathing air obtained from gas cylinders. After delivery, dams and pups were kept in the hypoxic chamber for another 3 days. The 4 control dams were placed in the normoxic condition and the oxygen level was in the range of 21% ± 1%. The control and hypoxic pups were used in quantification of gene expression.

Brain Tissue Collection

After hypoxia exposure, the brain was collected from 3 pups on PND 3. The dams and remaining pups were housed at 21% ± 1% oxygen and 3 pups were sacrificed on PND 8 for brain collection. Another 6 pups were from the control groups (n= 3 on PND 3 and n= 3 on PND 8). After live decapitation brains were rapidly removed and cortex was collected from control and hypoxic groups. Brain tissue was stored at -80 °C until analysis.

Purification of Total RNA

Total RNA was extracted using RNeasy mini kit (Qiagen, Frederick, MD, USA) according to the manufacturer's instructions. β-Mercaptoethanol (β-ME) was added to the supplied Buffer RLT as 10ul β-ME per 1ml Buffer RLT. The supplied Buffer RPE was added 4 volumes of ethanol to obtain a working solution. Brain tissue stored at –80°C was weighed on an electronic scale. The weighed frozen brain cortex (approximately 30 milligrams) was immediately placed in a liquid nitrogen-filled mortar and ground thoroughly with a pestle until the brain cortex turned into powder. After the liquid

nitrogen was evaporated completely, Buffer RLT with β-ME 600ul was added into the mortar. The mixture of brain tissue and Buffer RLT was ground until the mixture thawed, and then it was homogenized by passing the lysate at least 5 times through a blunt 20-gauge needle fitted to an RNase-free syringe. The lysate was centrifuged for 3 minutes at 13,300 rpm. The supernatant was removed carefully by pipetting and transferred to a new 1.5-mL microcentrifuge tube. One volume of 70% ethanol was added to the cleared lysate and mixed immediately by pipetting. The sample was transferred to an RNeasy spin column placed in a 2ml collection tube and was centrifuged for 15 seconds at ≥ 10,000 rpm. The flow-through was discarded. The above step was repeated until all the sample was transferred to the same RNeasy spin column. DNase I stock solution (Qiagen, Frederick, MD, USA) was used to remove the genomic DNA in the sample. The lyophilized DNase I (1500 Kunitz units in a vial) was dissolved in 550ul of the supplied RNase-free water using an RNase-free needle and syringe. The supplied Buffer RW1 (350ul) was added to the RNeasy spin column and was centrifuged for 15 seconds at ≥ 10,000 rpm to wash the spin column membrane. The flow-through was discarded. DNase I stock solution (10ul) was added to 70ul of the supplied Buffer RDD and mixed by gently inverting the tube. The tube was centrifuged briefly to collect residual liquid from the sides of the tube. The DNase incubation mix (80ul) was added directly to the RNeasy spin column membrane and was placed on the benchtop at room temperature for 15 minutes. The supplied Buffer RW1 (350ul) was added to the RNeasy spin column and was centrifuged for 15 minutes at ≥10,000 rpm. The flow-through was discarded. The supplied Buffer RPE (500ul) was added to the RNeasy spin column and was centrifuged for 15 seconds at ≥10,000 rpm to wash the spin column membrane. The flow-through

was discarded. The supplied Buffer RPE 500ul was added to the RNeasy spin column and was centrifuged for 2 minutes at ≥10,000 rpm to wash the spin column membrane. After centrifugation, the RNeasy spin column was removed carefully from the collection tube so that the column did not contact the flow-through. The RNeasy spin column was placed in a new 2ml collection tube and the old collection tube with the flow-through was discarded. It was centrifuged for 1 minute at 13,300 rpm to eliminate any possible carryover of Buffer RPE or residual flow-through remains on the outside of the RNeasy spin column. The RNeasy spin column was placed in a new 1.5ml collection tube. The RNase-free Millipore water (35ul) was added directly to the spin column membrane. The Rneasy spin column was centrifuged for 1 minute at ≥10,000 rpm to elute the RNA. The concentration of total RNA was measured in the NanoVue spectrophotometer (GE Healthcare, UK) at 260-nm absorbance. The purity of total RNA was determined by the ratio of the readings at 260nm and 280nm (A_{260}/A_{280}). A_{260} and A_{280} are the optical spectrometer measurement of absorbance at the wavelengths of 260nm and 280 nm respectively. A_{260} is frequently used to measure DNA/RNA concentration and A_{280} is used to measure protein concentration. A ratio of A260/A280 > 1.8 suggests little protein contamination in a DNA/RNA sample. In our study, only the sample of total RNA that has an A_{260}/A_{280} ratio of 1.9–2.1 and the concentration was above 250ng/µl was used to do the experiments.

Synthesis of cDNA

The cDNA-synthesis was performed by using the RT2 First Strand Kit (Qiagen, Frederick, MD, USA). An aliquot (2ug) of the total RNA was subjected to synthesize cDNA. All reagents were briefly (10-15 seconds) spin down before the experiment. The

Genomic DNA (gDNA) Elimination Mixture was prepared as described below. Total RNA 2.0ug, the supplied GE (5X gDNA Elimination Buffer) 2.0ul, and the Millipore water (RNase-free) were added together and the final volume of the mixture was up to 10.0ul. The contents were mixed gently with a pipettor followed by brief centrifugation. The mixture was incubated at 42^{o}C for 5 minutes and then chilled at 4 degree for at least one minute. The RT Cocktail was prepared as described below and all reagents were supplied. For one reaction, 5X RT Buffer 3 4ul, Primer and External Control Mix 1ul, RT Enzyme Mix 3 2ul, and RNase-free water 3ul were added together and the final volume was 10ul. In total, 12 reactions were prepared. RT Cocktail (10ul) was added to each 10ul-Genomic DNA Elimination Mixture to make First Strand cDNA Synthesis Reaction. The reaction was mixed gently with a pipettor. It was incubated at 42^{o}C for exactly 15 minutes and then was stopped immediately by heating at 95^{o}C for 5 minutes. The RNase-free Millipore water (91ul) was added to each 20ul of cDNA synthesis reaction and mix well. The finished First Strand cDNA Synthesis Reaction was stored at minus 20^{o}C until the next step.

Setup ABI 7500 Fast System

Please see Appendix 1.

Real-Time PCR for Innate and Adaptive Immune Response RT^{2} Profiler PCR Arrays

The RT^{2} SYBR Green/ROX PCR Mastermix (Qiagen, Frederick, MD, USA) and the cDNA synthesis reaction were briefly centrifuged for 10-15 seconds to bring the contents to the bottom of the tube. The PCR components mix was prepared in a loading

reservoir as described below. The RT^2 SYBR Green Mastermix (2X) (1350ul), cDNA synthesis reaction (102ul) and RNase-free Millipore water (1248ul) were added together, and the total volume was 2700ul. The Innate and Adaptive Immune Response RT^2 Profiler PCR Array (Qiagen, Frederick, MD, USA) was removed carefully from its sealed bag. PCR components mix 25ul was added to each well of the Array using an 8-channel pipettor. The Array was carefully and tightly sealed with Optical Adhesive Film. It was centrifuged for 1 minute at 1000g at room temperature to remove bubbles and then, was visually inspected from underneath to ensure no bubbles were present in the wells. The Array was placed in the real-time cycler and the run was started. Instructions for performing Real-Time PCR detection and the calculation of the threshold cycle (Ct) after the PCR run are found in Appendix1. When calculation of the Ct was completed, the Ct values were exported for all wells to a blank Excel spreadsheet for use with Web-based software from Qiagen, Frederick, MD, USA.

Statistical Analysis

For normalization of gene expression on the RT2 PCR Profiler PCR Array, five housekeeping genes, ribosomal protein large P1, hypoxanthine phosphoribosyltransferase 1, ribosomal protein L13A, lactate dehydrogenase A, and β-actin, were used. The C_t was calculated for each sample and normalized to the average C_t of the five housekeeping genes. A comparative C_t method was used to determine relative gene expression. The online data analysis software uses ΔΔCt method to do data analysis (please see Appendix 1). The significant results of fold change were not reported if the genes' threshold cycle had outliers or their threshold cycle was greater than the defined cut-off value (default 35). The p-value was calculated using a Student's t-test (two-tailed, equal variance) at the

0.05 significance level. The statistical analysis was performed using Web-Based RT^2 Profiler PCR Array Data Analysis version 3.5 (Qiagen, Frederick, MD, USA).

Results

The mRNA expression of innate and adaptive immune response was detected from hypoxic and control pups at PND3 (immediately after removal from hypoxia) and PND8 (five days after removal from hypoxia). In our study, 84 genes in total related to innate and adaptive immunity were tested in the two groups (hypoxia and control) at PND 3 and PND 8. In total, we found the expression of 14 genes was significantly (< 0.05) altered after induction of hypoxia in rats. The early stage, or primary insult, is the time when the animals were removed from hypoxia. During the primary insult, the mRNA expression of immune responses was compared between the hypoxic and control rats on PND 3. Nuclear factor kappa light polypeptide gene enhancer in B-cells 2 (*Nfkb2*), Toll interacting protein (*Tollip*), Tnf receptor-associated factor 6 (*Traf6*) were up-regulated in the hypoxic rats on PND 3 compared to PND 3 in the control rats (see Fig. 11). The recovery stage, or secondary insult, is 5 days after removal from hypoxia. During the secondary insult, the mRNA expression was compared between the hypoxic and control rats on PND 8. Interleukin 1 receptor-like 2 (*Il1rl2*), Interleukin 1 receptor antagonist (*Il1rn*), Peptidoglycan recognition protein 1 (*Pglyrp1*), Platelet-activating factor receptor (*Ptafr*), Toll-like receptor 1 (*TLR1*) were up regulated in the hypoxic rats on PND 8 compared to the control rats on PND 8 (see Fig. 12). The progressive stage, or secondary insult, is 5 days after removal from hypoxia and we evaluated the progressive changes of immune responses following hypoxia. At this stage, gene expression of immune responses was compared in the hypoxic group between PND 3 and PND 8.

Interleukin 1 alpha (*Il1a*), Platelet-activating factor receptor (*Ptafr*), Toll-like receptor 6 (*TLR6*), Toll-like receptor 9 (*TLR9*), Tumor necrosis factor (TNF superfamily, member 2) (*Tnf*), Toll interacting protein (*Tollip*) were up-regulated on PND 8 compared to PND 3 in hypoxic rats, however, the gene of Adenosine A2a receptor (*Adora2a*), and Chemokine (C-X-C motif) receptor 4 (*Cxcr4*) were down-regulated (see Fig. 13). There was one significant down-regulation of gene expression between PND 3 and PND8 in the control group, Chemokine (C-X-C motif) receptor 4 (*Cxcr4*) (see Fig. 14).

Discussion

The data support our hypothesis that gene expression of immune responses is activated after perinatal hypoxia. The mRNA expression of immune responses and their related pathways in brains were different at the two time points (PND 3 and PND 8) between the control group and hypoxic group. Previous research indicates most kinds of brain pathophysiological processes, such as neonatal HI, trauma brain injury, stroke, neurodegenerative diseases, even stress, can cause immune responses (Liu et al., 2012; Chen et al., 2011; Wang et al., 2007; Shrivastava et al., 2013; Pradhan & Andreasson 2013; Lavretsky & Newhouse, 2012). The up-regulation of *IL-1* and *TNFα* is generally consistent with studies on other types of brain injury, such as neonatal hypoxia-ischemia and stroke, which induce the increase of expression of cytokine genes, *IL-1* and *TNF-α* (Hedtjarn et al., 2004; Wang et al., 2007). Some genes not previously reported in the literature on neonatal HI and traumatically brain-injured (TBI) were also identified, including *Tollip, Il1rl2, Pglyrp1, and TLR6*. The use of different models (neonatal HI and cerebral ischemia vs perinatal hypoxia) may explain the differences found between studies. In our study, we focused on a lack of oxygen in the developing brain during the

perinatal period of the rat. The neonatal hypoxia-ischemia model, however, often includes oxygen deprivation with mechanically reduced cerebral blood flow and the cerebral ischemic model includes occluding the middle cerebral artery permanently or transiently in adult animals. We report changes in genes related to innate and adaptive immunity after perinatal hypoxia, including *Tollip, TRAF6, Il1rl2, Pglyrp1, and TLR6,* for the first time.

NF-κB2 was up-regulated in the hypoxic rats at PND 3 compared to PND 3 in the control rats. *NF-κB* is one kind of transcription factors, which regulates downstream gene expression. This transcription factor can translocate to the nucleus and bind to its specific sites. These specific domains within the promoters of downstream genes can activate their transcription (Wang et al., 2007). It is now well recognized *NF-κB* is involved in the regulation of inflammation (Baeuerle and Henkel, 1994). Increased expression of *NF-κB* has been found after neonatal HI (Northington et al., 2011). Decreased expression of *NF-κB* coincidental with inflammatory cytokines such as *IL-1β*, *IL-6*, and *TNF-α* has also been found after treatment with a neuroprotective drug in the same study of neonatal HI. Inhibition of *NF-κB* has been found to improve long-term motor and cognitive outcome after neonatal HI in rats (van der Kooij et al., 2010). Some inflammatory genes contain functional κB sites, such as tumor necrosis factor-α (*TNF-α*) and interleukin-6 (*IL-6*) (Wang et al., 2007). *NF-κB* is an important transcription factor and may participate in the production of inflammatory cytokines after neonatal hypoxia-ischemia. Although neonatal HI and perinatal hypoxia are different, they may have similar pathophysiological processes, especially immune responses. *NF-κB* is considered part of the immune response and it may have a close relationship with inflammatory cytokines after perinatal

hypoxia. The genes of inflammatory cytokines were not expressed in this stage, which may be due to other regulatory mechanisms, such as the immunosuppression of glucocorticoids (McEwen et al., 1997; Turnbull & River, 1999). Inflammatory cytokines may primarily contribute to secondary brain injury after hypoxia since the gene expression of cytokines was not detected in the early brain injury. The up-regulation of transcription factor *NF-κB* in our study indicates inflammatory pathways may have been activated after perinatal hypoxia.

Toll interacting protein (*Tollip*) was up-regulated in the hypoxic rats compared to the control rats at PND 3. *Tollip* represents one of the endogenous modulators of *Toll-like receptors* (*TLRs*) that are involved in innate immunities (Capelluto, 2012). The activation of *TLRs* by pathogen or damaged tissues can trigger a specific *TLR*-mediated downstream signaling. *Tollip* modulates the process of *TLRs*-mediated signaling. Several studies have showed *Tollip* inhibits *TLR*-mediated immune responses (Bulut et al., 2001; Zhang & Ghosh, 2002). Overexpression of *Tollip* results in inhibition of *TLR2*, *TLR4*, and *IL-1R* signaling in *in vitro* studies (Zhang & Ghosh, 2002; Burns, et al., 2000; Brissoni et al., 2006; Piao et al., 2009). However, recent study in *Tollip*-deficient mice has shown the reduced production of inflammatory cytokines, such as *TNFα*, *IL-6*, and *IL-1β* (Didierlaurent et al., 2006). The above studies confirm the modulatory function of *Tollip* in immune responses. Until now, there have been no reports in the literature to show the effects of *Tollip* on modulating inflammation under the conditions of perinatal hypoxia. It is unknown whether *Tollip* inhibits or improves the immune responses and the production of inflammatory cytokines after perinatal hypoxia. *Tollip* may play a modulatory role (inhibition or improvement of the immune responses) depending on the time progression

of hypoxic brain injury. The up-regulation of *Tollip* may also relate to the alteration of gene expression of *TLR1, TLR6, and TLR9* on the other time point after hypoxia because its function is to modulate the process of *TLRs*-mediated signaling. The up-regulation of *Tollip* in our study suggests innate immune responses may be involved in neonatal hypoxia.

Tnf receptor-associated factor 6 (*TRAF6*) was up-regulated in the hypoxic rats compared to the control rats at PND 3. *TRAF6* is an adaptor protein of the tumor necrosis factor receptor-associated factor family and it is the only member that mediates both tumor necrosis factor receptor and *IL-1* receptor/*TLR* signaling (Liu et al., 2010). Several studies have been shown the expression of *TRAF6* is increased after brain injury such as TBI and cerebral ischemia (Liu et al., 2012; Chen et al., 2011). Liu et al. (2012) found *TRAF6* positive cells and the protein levels of *TRAF6* were significantly increased in the ischemic cortex 24 hours after the induction of permanent MCAO in rats and these increases were reduced after the treatment with a neuroprotective drug. The protein expression of *TRAF6* was also increased 7 days after TBI in adult rats and was mainly expressed in the ipsilateral injured brain (Chen et al., 2011). One *in vitro* study of hypoxia on neurons found *TRAF6* expression was up-regulated during hypoxia (Frøyland et al., 2008). However, these above studies of *TRAF6* expression did not mention whether it was associated with inflammation. *TRAF6* may relate to inflammation after brain injury and hypoxia based on its gene function. Unfortunately, no previous research outlines the roles of *TRAF6* in perinatal brain injury or perinatal hypoxia. We speculate *TRAF6* may be involved in the immunity and it mediates *TNF* receptor, *IL-1* receptor/*TLR* signaling, in which transcription factor *NF-κB* is also involved. The up-regulation of *TRAF6* may

explain the alteration of gene expression of *Tnf, Ilr1, TLR1, TLR6, and TLR9* on the other time points after hypoxia because *TRAF6* regulates expression of these genes. In our study, we suggest the up-regulation of *TRAF6* may mediate inflammatory processes during perinatal hypoxia.

In the primay injury, *NF-κB*, *Tollip*, and *TRAF6* are all in inflammatory pathways. At this stage, gene expression of inflammatory cytokines is not significantly up-regulated. The three genes have effects on mediation or regulation of immune responses. The increase of gene expression at this stage suggests inflammatory pathways are activated and may be prepared for producing cytokines. The lack of up-regulation of cytokines in the early stage indicates inflammatory cytokines may mainly contribute to the secondary brain damage after hypoxic exposure.

Interleukin 1 receptor-like 2 (*Il1rl2*) was up regulated in the hypoxic rats compared to the control rats at PND 8. *Il1rl2* was first identified in 1996 (Lovenberg et al., 1996), indicating it is a relatively novel receptor. *Il1rl2* binds the novel *IL-1* family cytokines that are interlukin-1 family member-6 (*IL-1F6*), *IL-1F8*, and *IL-1F9* (Towne et al., 2004). These *IL-1*-like cytokines are involved in inflammation. These cytokines do not bind the classic *IL-1* receptor, binding instead to the *IL-1 receptor-like 2* to signal inflammatory information (Ramadas et al., 2011). An investigation of the literature indicates no study of the effects of *Il1rl2* on brain injury has been reported until now. In our study, the fold change of *IL-1F8,* which binds to *Il1rl2*, was also increased, although the change was not significant. The increased expression of *IL-1F8* and its receptor *Il1rl2* indicates a novel inflammatory pathway may be involved in brain injury after perinatal

hypoxia. In our study, the up-regulation of *Il1rl2* suggests a novel inflammatory pathway may participate in the process of secondary brain injury after perinatal hypoxia.

Interleukin 1 receptor antagonist (*Il1rn*) was up regulated in the hypoxic rats compared to the control rats at PND 8. Il1rn inhibits the inflammatory effects of *IL-1α* and *IL-1β*, so it has anti-inflammatory effects. One animal study demonstrated administration of recombinant *Il1rn* (intracerebroventricular injection 30 minutes before and 10 minutes after cerebral ischemia) attenuates the infarct area and neuronal death 24 hours after focal cerebral ischemia in adult rats (Relton & Rothwell, 1992). In another animal study (neonatal HI model) administration of recombinant *Il1rn* by subcutaneous injection also reduced the weight of the lesioned area and neuronal death in the striatum (Martin et al., 1994). These studies indicate *Il1rn* has neuroprotective effects, which may result from the anti-inflammatory function of *Il1rn*. Neonatal HI caused down-regulation of Il1rn concomitant with up-regulation of *IL-1β* (e.g. Girard et al., 2008) and administration of *Il1rn* reduced neural cell death (Hu et al., 2005). The up-regulation of gene expression of *Il1rn* in our study is not consistent with previous study of down-regulation of *Il1rn* after neonatal HI. Different models of brain injury were used and gene expression was examined at different times after the induction of models between our study and the previous study, which may have led to different results. For example, neonatal HI rat model was in the literature (Girard et al., 2008), but perinatal hypoxic rat model was used in our study. In the literature, gene expression was examined at 4hs, 18hs, 24hs, and 40hs post-HI (Girard et al., 2008), but gene expression was examined at 0 day and 5 days post perinatal hypoxia in our study. Our study indicates *Il1rn* is involved in the immune responses after perinatal hypoxia. During the secondary insult, in addition

to the activation of inflammatory cytokine receptors such as *Il1rl2*, the anti-inflammatory gene *Il1rn* was also initiated, which may neutralize the detrimental effects of inflammation (McCombe & Read, 2008). In the present study, the up-regulation of *Il1rn* that occurred 5 days after hypoxia exposure indicates anti-inflammatory mechanisms, which normally are recruited to fight with the immune reactions, may be activated and may help protect the brain from perinatal hypoxia.

Peptidoglycan recognition protein 1 (*Pglyrp1*) was up regulated in the hypoxic rats compared to the control rats at PND 8. *Pglyrp1* is a ubiquitous protein involved in innate immunity. It has been well studied that *Pglyrp1* has antibacterial activities (Cho et al., 2007; Guan et al., 2004) and immunomodulatory activity (Saha et al., 2009). Saha et al. (2009) found *Pglyrp1* had anti-inflammatory properties to limit over-responsiveness of the immune system to bacteria in the study of development of arthritis in mice. There is no previous evidence in the literature to show effects of *Pglyrp1* on any kind of brain injury. In our study, increased expression of *Pglyrp1* indicates *Pglyrp1* might be another anti-inflammatory factor. Our increase in *Pglyrp1* may protect the brain from further injury induced by inflammatory cytokines after perinatal hypoxia.

Platelet-activating factor receptor (*Ptafr*) was up regulated in the hypoxic rats at PND 8 compared to the control rats at PND 8. Perinatal hypoxic insult causes the increase of lipid peroxidation products (Rogers et al., 1997; Schmidt et al., 1996), which may be associated with the production of *PAF*. In term infants with perinatal asphyxia, plasma *PAF* concentrations were higher than in normal full term infants (Akisu et al., 1998). *PAF* may contribute to neuronal injury by increasing intracellular calcium concentrations and by stimulating production and release of pro-inflammatory cytokines

(Doucet & Bazan, 1992). *Ptafr* plays a role in mediating these effects of *PAF*. *PAF* binding to Ptafr triggers a variety of intracellular signaling cascades and initiate or amplify inflammatory and/or apoptotic events (Maerz et al., 2011). An investigation of the literature indicates no study of the effects of *Ptafr* on brain injury has been reported. Our study indicates *Ptafr* may have effects on inflammation and secondary progression of hypoxic injury and may participate in and even amplify the process of inflammation induced by perinatal hypoxia.

Toll-like receptor 1 (*TLR1*) was up regulated in the hypoxic rats on PND 8 compared to the control rats on PND 8. *TLRs* are key components of the innate immune system. Usually *TLRs* are recognized to have their roles in bacterial and viral infection, but recent studies have shown *TLRs* are also associated with brain injury, especially after neonatal HI exposure (Kariko et al., 2004; Lehnardt et al., 2008; Park et al., 2004). One study of neonatal mice after HI found gene expression of *TLR1* was up-regulated at 6 hours and 24 hours after HI, but the *TLR1* positive cells were found only at 24 hours, not 6 hours after HI (Stridh et al., 2011). The brain infarct area was not decreased in *TLR1* knockout mice compared to wild type mice (Stridh et al., 2011), which meant that *TLR1* did not play a critical role in neonatal brain injury. The result of up-regulation of *TLR1* in the hypoxic brain from our study is consistent with the study of neonatal mice brain after hypoxia/ischemia. The up-regulation of *TLR1* in our study indicates innate immune system may participate in the process of brain injury and regulate inflammation after perinatal hypoxia.

During the secondary insult, genes expression of immune modulators, inflammatory cytokine receptor, and anti-inflammatory factor was up-regulated.

Although the hypoxic animals were taken out of the hypoxic chamber at this stage, immune responses induced by neonatal hypoxia had continuous effects on brain. Thus, immune responses may contribute to the secondary insult on the developing brain. Another important characteristic at this stage is that gene expression of anti-inflammatory mechanisms is also activated, which indicates recovery mechanisms may be initiated.

The most notable gene changes were found in the hypoxic group between PND 3 and PND 8. Interleukin 1 alpha (*Il1a*) what was up-regulated on PND 8 compared to that on PND 3 in hypoxic rats. *Il1α* is one of the pro-inflammatory cytokines of the *IL-1* family. *Il1α* triggers inflammation in a pathway initiated through MyD88 activation and culminated in *NF-κB*-induced transcription of inflammatory genes (Rider et al., 2011). *Il1α* is a "dual-function" cytokine, binding to its respective cell surface receptor to mediate extracellular effects (Dinarello, 2011). The intracellular precursor forms translocate to the nucleus and influence transcription of pro-inflammatory genes. Rider et al. (2011) reported the role of *Il1α* in a mouse model of sterile inflammation, which was associated with tissure injury. They found *Il1α* released from necrotic cells initiated inflammation by the infiltration of neutrophils. A study of neonatal hypoxic/ischemic brain injury found *Il1α* was increased in the serum of rats two days after the induction of hypoxia/ischemia (Rosenkranz et al., 2012). In turn, application of umbilical cord blood cells reduced the serum level of *Il1α*. In the present study, the up-regulation of gene expression of *Il1α* in the developing brain following the induction of perinatal hypoxia is consistent with the study by Rosenkranz, 2012. *Il1α* may have effects on initiating inflammation 5 days after the induction of perinatal hypoxia. The up-regulation of *Il1α*

indicates immune responses or inflammation may have effects on the process of secondary brain injury after hypoxic exposure.

Toll-like receptor 6 (*TLR6*) was up-regulated on PND 8 in hypoxic rats compared to that on PND 3. They have roles in bacterial and viral infection and in addition, they are associated with brain injury especially after hypoxic/ischemic exposure. In the literature, *TLR2* and *TLR4* are heavily studied in neuropathological processes. *TLR6* is one of the Toll-like receptor members so it may have a role in regulation of inflammation after induction of hypoxia. One *in vitro* study showed *TLR6* and *TLR2* gene expression was significantly up-regulated after hypoxic exposure in different cells types and cell-lines, including human dendritic cells, monocytic cells (MM6), endothelia (HMEC-1) or intestinal epithelia (Caco-2) (Kuhlicke et al., 2007). This *in vitro* study of *TLR6* indicates innate immune responses may be involved in the process of hypoxia in different cell lines. Literature investigation indicates no *in vivo* study describes the function of *TLR6* during the process of brain injury. Thus, the function of *TLR6* in brain injury is not clear. The result of increased expression of *TLR6* in the current *in vivo* study is generally consistent with the previous *in vitro* study (Kuhlicke et al., 2007). Our result demonstrates innate immune system may be involved in injury induced by hypoxia, not only *in vitro*, but also *in vivo*. Our study indicates innate and adaptive immunity may regulate non-infectious inflammation, which may be involved in the progression of brain injury induced by perinatal hypoxia.

Toll-like receptor 9 (*TLR9*) was up-regulated at PND 8 in hypoxic rats compared to that at PND 3. *TLR9* is another member in *TLRs* family and is expressed in rodents and humans. One study of inflammation responses in spinal cord injury showed *TLR9* was

associated with sterile inflammation after traumatic spinal cord injury (David et al., 2013). In TBI mice, there was no *TLR9* alteration, but *TLRs 1* and *2* were up-regulated (Hua et al., 2011). Exogenous progesterone treatment is thought to inhibit the increase of some inflammatory cytokines and inflammation-related factors induced by TBI. After treatment with progesterone, *TLRs 1* and *2* were not down-regulated, *TLR9* was up-regulated, but some of the cytokines were down-regulated (Hua et al., 2011). The TBI study by Hua et al. (2011) demonstrates how TLRs regulate inflammatory cytokines. Although the role of *TLRs* is still unclear, the above studies of traumatic spinal cord injury and TBI indicate *TLR9* may reduce or increase the expression of cytokines under specific conditions. Several studies showed neuroprotective effects against brain ischemic injury were induced by preconditioning with *TLR9* ligand CpG oligodeoxynucleotide (Packard et al., 2012; Stevens et al., 2008). One *in vitro* study found neuronal injury was mediated by stimulation of microglial *TLR9* (Iliev et al., 2004). Although there is no study that has been done on the effects of *TLR9* on brain injury induced by perinatal hypoxia, the up-regulation of *TLR9* in our study indicates it may regulate the process of inflammation after perinatal brain injury. It is possible that *TLR9* may cause neuronal injury by inducing inflammation or alternatively it may play a role in decreasing expression of cytokines. In our study, three *TLRs (TLR1, TLR6, and TLR9)* were up-regulated after perinatal hypoxia, indicating *TLRs* or the innate immune system may modulate the progression of brain injury induced by perinatal hypoxia. How *TLRs* modulate perinatal brain injury requires future research to be fully understood. The up-regulation of *TLR9* demonstrates innate immune responses may have effects on the secondary brain damage induced by hypoxia.

Tumor necrosis factor superfamily member 2 (*TNF*) was up-regulated on PND 8 compared to that on PND 3 in hypoxic rats. Tumor necrosis factor (also named as tumor necrosis factor alpha and tumor necrosis factor superfamily member 2) is widely expressed in the brain where it appears to be produced largely by glial cells (Breder et al., 1993). Two different Tnf receptors have been identified p55 (or p60) and p75 (or p80) (Armitage, 1994; Beutler & Van Huffel, 1994). *TNF-α* is a member of a cytokine family.

One study found the protein expression of *TNF-α* in the cerebellum was enhanced after neonatal hypoxia exposure in rats (Kaur et al., 2012). In the same study, cultured microglia released significantly high levels of *TNF-α* after hypoxia exposure compared to control cultured microglia. *TNF-α* induced neuronal death in cultured Purkinje neurons when treated with *TNF-α* (Kaur et al., 2012). Another study has shown *TNF-α* is associated with attenuating synaptic transmission after hypoxia exposure in rat hippocampal slices (Batti & O'Connor, 2010). In contrast, other studies demonstrated *TNF-α* has neuroprotective effects after ischemia/reperfusion injury (Bruce et al., 1996; Cheng et al., 1994; Goel et al., 2010). Neuronal death and oxidative stress caused by focal cerebral ischemia was exacerbated in *TNF* receptor knockout mice (Bruce et al., 1996). In cultured embryonic rat hippocampal, septal, and cortical neurons, *TNF-α* reduced neuronal death and promoted calcium homeostasis after glucose deprivation-induced injury (Cheng et al., 1994). *TNF-α* decreased the levels of pro-apoptotic protein, but increased the levels of anti-apoptotic protein in cortical neurons after hypoxia exposure (Goel et al., 2010). The two opposing effects of *TNF-α* depend on the specific activation of one of the two Tnf receptors and the differential downstream pathway activated, and also relate to its concentration in the injury area and time of exposure

(Bernardino et al., 2005; Markus et al., 2009; Scherbel et al., 1999). Tnf receptor expression was found in injured rat brain following perinatal asphyxia, but *TNF-α* did not play an important role in astrogliosis (Maslinska et al., 2002), indicating *TNF-α* is involved in part of the immune response. *TNF-α* has also been found to be significantly increased after perinatal asphyxia in humans (Foster-Barber et al., 2001). The up-regulation of *TNF-α* in the present study is generally consistent with the previous animal and human studies.

Although the role of *TNF-α* in brain injury after perinatal hypoxia is not very clear, it is assumed that *TNF-α* may have dual roles in brain injury after perinatal hypoxia. Both opposing effects of *TNF-α* might affect the neonatal brain after the induction of hypoxia, depending on the amount of time that has passed hypoxia exposure. If the *TNF-α* plays a beneficial role, the up-regulation of *TNF-α* indicates repair mechanisms may be activated after hypoxic brain injury. In contrast, if the *TNF-α* has detrimental effects on brain injury, the up-regulation of *TNF-α* demonstrates inflammatory cytokines may contribute to the secondary damage after perinatal hypoxia. Further studies are required to examine the function of *TNF-α* in brain injury after exposure to perinatal hypoxia. In our study, the up-regulation of *TNF-α* may have either beneficial or destructive effects on the developing brain after hypoxic exposure based on the previous studies mentioned above.

The gene of Adenosine A2a receptor (*Adora2a*) was down-regulated in hypoxic animals between PND 3 and PND 8. Adora2a has two important functions, one is exerting immune-modulating effects in immune responses after whiter matter lesions induced by chronic cerebral hypoperfusion (Duan et al., 2012) and the other is facilitating

hypoxia/ischemia-evoked release of excitatory amino acids (Ongini et al., 1997). Several studies have shown the activation of Adora2a protects against inflammatory damage through inhibition of pro-inflammatory cytokine production in immune and inflammatory cells in animal models of several injuries, such as intracerebral hemorrhage (Mayne et al., 2001), renal ischemia (Day et al., 2003), and hypoxia in local tissues (Sitkovsky et al., 2004). In contrast, Chen & Pedata (2008) reviewed the Adora2a modulation of neuroinflammation may differentially affect the outcome of brain injury depending on the nature of brain injury. In the literature, some studies have shown the beneficial effects of Adora2a following brain injury, such as whiter matter lesions (Duan et al., 2012) and neonatal HI (Aden et al., 2003) in mice. However, other studies have shown Adora2a aggravated brain damage in an animal model of cerebral ischemia (Ongini et al., 1997) and an Adora2a antagonist reduced brain injury in neonatal HI in rats (Bona et al., 1997). The roles of Adora2a in the prenatal hypoxic brain injury are less clear-cut. The outcome of down-regulation of *Adora2a* in our study is not known. The function of Adora2a may be related to the nature of perinatal hypoxia and the time period after perinatal hypoxia. Further research is required to understand the role of Adora2a in perinatal brain injury. We suggest the down-regulation of *Adora2a* may result in decreased release of excitatory amino acids, or it may lead to increased production of pro-inflammatory cytokines.

The chemokine (C-X-C motif) receptor 4 (*Cxcr4*) gene was down-regulated in hypoxic animals between PND 3 and PND 8. Cxcr4 is a G protein-coupled receptor for the chemokine stromal–cell-derived factor-1 (*SDF-1*) and it may have a role in brain development (NCBI Gene). Cxcr4 is the primary transmembrane receptor for signaling chemokine *SDF-1*. The binding of *SDF-1* to *Cxcr4* is involved in a variety of

physiological and pathological events including blood homeostasis (Baggiolini, 1998), cellular inflammation and immune response (Aiuti et al., 1999), tumor metastasis (Majka et al., 2000), and cell recruitment in injured tissues (Kitaori et al., 2009). Several studies reveal *SDF-1* and its receptor *Cxcr4* are up-regulated in the ischemic penumbra regions to induce an immune response following ischemic stroke, in contrast, *SDF-1* and *Cxcr4* also play a role in neurovascular repairing (reviewed in Wang et al., 2012). Itoh et al. (2009) found *Cxcr4* expression was also up-regulated after traumatic brain injury in rats and it had the potential abilities to improve the brain dysfunction after traumatic brain injury. Similarly, one *in vitro* study of cultured human NT2-N neurons showed a significant increase in RNA of *Cxcr4* right after hypoxia and glucose deprivation (Frøyland et al., 2008). *SDF-1* and *Cxcr4* system also has effects on guiding innate neural stem cells and transplanted umbilical cord blood cells to the damaged areas and their maturation (Itoh et al., 2009; Rosenkranz et al., 2010). Taken together, the data indicate *Cxcr4* has two opposite functions. Immune response induced by up-regulated *Cxcr4* plays a destructive role in brain injury, but up-regulation of *Cxcr4* initiates neurovascular generation and guiding neural stem cells migration, which benefits brain injury.

The down-regulation of gene expression of *Cxcr4* in the present study is not consistent with previous research in which *Cxcr4* expression was up-regulated after traumatic brain injury in rats (Itoh et al. 2009) and hypoxia and glucose deprivation in cultured neurons (Frøyland et al., 2008). The main reason is likely that different animal models are used in our study compared to previous studies. The other reason is that tissue collection was performed at different time points (5 days after hypoxia) in our study compared to previous studies (3-7 days after TBI), which may result in different gene

expression of *Cxcr4* due to time point of tissue collection. *Cxcr4* may activate immune response by its own pathway or chemotaxis in hypoxic brain injury and result in brain damage. *Cxcr4* has two functions after brain injury, inflammation and neurogeneration. In our study, the down-regulation of *Cxcr4* indicates a potential survival mechanism may be initiated by decreasing the immune responses or *Cxcr4* may also have negative effects on brain repair after perinatal hypoxia. The role of *Cxcr4* expression after perinatal hypoxia remains to be determined.

The other role of *Cxcr4* in the brain development cannot be ignored. At this specific period, the developmental factor must be taken into account. The significant down-regulation of Cxcr4 may relate to brain development as the same direction of regulation was found at the same period in the control group. The down-regulation of *Cxcr4* in the present study suggests either survival or detrimental roles in brain injury after perinatal hypoxia and *Cxcr4* may also regulate brain development.

During the secondary insult, gene expression related to immune responses has its specific characteristics. Firstly, notable changes in gene expression of immune responses occurred, which means that immune responses contribute mainly in the process of secondary brain injury. Secondly, recovery or tissue-protecting mechanisms may also occur. For example, increased expression of *Tnf-α* may have neuroprotective effects and decreased expression of *Adora2a* and *Cxcr4* may inhibit immune responses. Thirdly, one gene related to development plays a role in this period, as well. Inflammatory responses, anti-inflammatory and developmental mechanisms exert effects on secondary brain injury, indicating with increased maturity and longer recovery time after hypoxia, multiple factors contribute to the process of brain injury.

In our study, the time period of immune reactions responding to perinatal hypoxia is generally consistent with studies found in the literature. The gene expression of inflammatory cytokines and pathways is usually detected around 2-3 hours after hypoxia-ischemia (HI) in the developing brain (Hedtjärn et al., 2004; Shrivastava et al., 2013), but Stridh et al., (2011) found gene expression of some of the TLRs responded 30 minutes after neonatal HI. The immune responses are not limited to immediately after the brain injury and they last a few days. Hedtjarn et al. (2004) showed gene expression of inflammation was still detected 3 days after HI. Shrivastava et al. (2013) showed inflammatory cells such as activated astrocytes and microglia/macrophages were seen till 7 days after hypoxia, indicating the inflammation was lasting after hypoxia/ischemia. In our study, we found immune responses were identified on the last day animals were in the hypoxic chamber and some of the responses were still detected 5 days after hypoxia. The results of our study are generally consisitent with other studies of neonatal brain injury, in which immune responses were found between 2-3 hours and 7 days after brain injury (Stridh et al., 2011; Hedtjärn et al., 2004; Shrivastava et al., 2013). Our results indicate the response of the immune system is fast and out lasts the hypoxia.

In addition to causing brain damage, inflammation also have a protective role during brain injury and are involved in repair process after cerebral ischemia (McCombe & Read, 2008). During primary insult, activated microglia may exert neuroprotection by producing neurotrophic molecules, such as brain-derived neurotrophic factor (BDNF), insulin-like growth factor I (IGF-I), and several other growth factors (Lakhan et al., 2009) and by phagocytic function (Raivich et al., 1999). During the secondary insult, anti-inflammatory cytokines are released from the immune cells and activated T cells can also

produce neurotrophins, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and neurotrophins 4 and 5 (NT-4 and NT-5) (McCombe & Read, 2008). Thus, the role of inflammation and immune responses in cerebral ischemia is twofold. We assume immune responses would participate in the repair process after perinatal hypoxia. In the present study, we did not find gene expression of anti-inflammatory cytokines and we did not test the expression of neurotrophins, but we did find other neural repair mechanisms after perinatal hypoxia. Up-regulation of *TNF-α* during the secondary insult (5 days after hypoxia) may play a role in brain recovery. Two other genes that are associated with reducing inflammation were altered in our study. One pro-inflammatory cytokine receptor antagonist, interlukine-1 receptor antagonist, was up-regulated and one chemokine receptor, chemokine (C-X-C motif) receptor 4, was down-regulated. The data in our study are consisitent with the findings that immune responses are twofold (Vexler & Yenari, 2009). The balance between the ability of an immune response to cause damage and to participate in repair determines the outcome of injury.

In the present study, some of the classic markers of brain injury were altered, including IL-1α and TNFα, but others were not altered, such as IL-6, TLR2, and TLR4 (Du et al., 2011; Hua et al., 2011; Stridh et al., 2011; Hyakkoku et al., 2010; Caso et al., 2007). These differences may be due to different models of brain injury used. The animal model induced in our study was perinatal hypoxia using a depletion of oxygen in the breathing air. In the literature, authors used other kinds of animal models, including neonatal hypoxia-ischemia (Stridh et al., 2011), middle cerebral artery occlusion (Caso et al., 2007), or traumatic brain injury (Hua et al., 2011).

The method of induction of neonatal HI typically used requires one side of the common carotid artery to be ligated for approximately 5 minutes and then the animals are placed in a hypoxic incubator with a 10% oxygen level for 1-2 hours (Stridh et al., 2011). Reduced cerebral blood flow followed by lack of oxygen during the process of hypoxia-ischemia can result in severely decreased delivery of oxygen and energy substrates to the brain. It is then followed by reperfusion characterized by deleterious effects of reoxygenation. Many studies have shown animals have obvious behavioral deficits and an infarct area in the brain after neonatal hypoxia-ischemia (Stridh et al., 2011; Pimentel et al., 2009; Karalis et al., 2011). In the adult rodent model of MCAO, the middle cerebral artery is occluded either for 2 hours or permanently. The longer time of decrease in cerebral blood flow followed by reperfusion can result in cerebral infarct and neurological deficits (Hyakkoku et al., 2010; Caso et al., 2007). Traumatic brain injury can result in a cascade of events initialize with a primary neuronal/glial insult and followed by proximal and distal cell loss (Hua et al., 2011). The pathological alterations in the hypoxic animal model used in our study are milder than in the model of neonatal hypoxia-ischemia, middle cerebral artery occlusion, and traumatic brain injury. Hypoxia exposure may not always produce obvious neuropathological changes, but they are visible at the microscopic and biochemical level (Grow & Barks, 2002). Severe, systemic hypoxia can also depress organ function, such as cardiac, liver and digestive systems and may affect cerebral perfusion and input of energy substrates to the brain. A study in neonatal animals demonstrates chronic neonatal hypoxia (9.5% O_2 for 30 days) decreases the volume of cortex and white matter, with altered production and maintenance of glial and neuronal cells of cerebral cortex (Schwartz et al., 2004). The study by Schwartz et al.

(2004) indicates long time neonatal hypoxia may cuase severe cortex injury. A relatively short time hypoxia (5 days hypoxia in our study) may result in mild brain injury. The mild brain injury after hypoxic exposure in our study may be the main reason why we did not obtain significant alteration of gene expression of other classic markers of brain injury, including *IL-6*, *TLR2*, and *TLR4*.

Although we did not find changes of gene expression in *TLR2* and *TLR4*, we did find other *TLRs* (*TLR1*, *TLR6*, and *TLR9)* were up-regulated after perinatal hypoxia. The up-regulation of *TLR1, 6,* and *9* suggest TLR signaling pathways may play a role after perinatal hypoxia. After TLR ligands bind to *TLRs*, TLR signaling pathways can be activated (Takeda & Akira, 2004). MyD88, an adaptor, binds to the receptor. Upon stimulation, IL-1 receptor-associated kinase and *TRAF6* are recruited to the receptor. Then, a series of kinases, such as Iκk and MAPK, are further activated, which induces the activation of transcription factors, such as *NF-κB*. The activation of transcription factors leads to the production of inflammatory cytokines (e.g. *IL-1* and *TNFα*). In addition to the molecules described above, *Tollip* is also implicated in TLR signaling pathways, which regulates these pathways. In our study, *TLR1, 6, and 9, Tollip*, *TRAF6*, *NF-κB, IL-1*, and *TNFα* were up-regulated, which are in TLR signaling pathways. Our results indicate TLR pathways may be involved in the perinatal brain injury after hypoxic exposure, which is consistent with the common acknowledgement that TLR pathways are associated with the immune responses after brain injury.

Our study investigated gene expression of immune responses after perinatal hypoxia in rats and the expression of 14 genes was significantly altered. Our data indicate immune responses are involved in the pathological processes of hypoxia. In addition to

inflammation, anti-inflammatory mechanisms are also activated after perinatal hypoxia. These anti-inflammatory mechanisms may account for only minimally impaired behavioral performance in rats. Deep understanding of the relationship between inflammatory mechanisms and anti-inflammatory mechanisms after perinatal hypoxia will allow researchers to manipulate these responses and discover effective neuroprotective treatment. The 14 genes may have the potential to be biomarkers of hypoxia. These genes are mainly expressed in the secondary phase of hypoxia (5 days after hypoxia), but not in the early phase (right after hypoxia), indicating some immune responses may contribute to secondary damage after hypoxia, and some may be involved in neuroprotection.

Chapter 5

Overall Discussion

Hypoxia occurs when there is abnormally low oxygen in the body, which has harmful effects on vital organs, such as the brain. Literature has shown fetal hypoxia can impair the developing brain (Tong & Zhang, 2012). Immune responses (Stridh et al., 2011; Hedtjarn et al., 2004) and stress hormones (Mandyam et al., 2008) are involved in the neonatal/prenatal brain injury, but little is known about the effects of these two factors with perinatal hypoxia in rats. Spontaneous behavioral tests can be used to determine whether normal developmental milestones are delayed, reflecting brain development. Whether spontaneous behavior is delayed following perinatal hypoxia in rats has not been determined. The goal of this research was to investigate the spontaneous behavioral development and the responses of the immune system of the brain and stress hormones with perinatal hypoxia in a rat model.

In the study described in Chapter 2, we conducted multiple spontaneous motor behavioral tasks to evaluate the brain development after perinatal hypoxia. Delayed ambulating behavior was found at the early age (PND 4 and PND 8) in the hypoxic animals. When examining righting behavior, the control rats used more right sides to roll onto the quadruped position at the old age (PND 10), not at the early age. In the hypoxic animals, there was a decreased total distance traveled at PND 11 and an increased total distance traveled at PND 21, which both approached significance. The differences between the hypoxic group and the control group found in the above three behavioral tasks (ambulating behavior, the side preference in right behavior, and total distance traveled) indicate the hypoxic animal had delayed or impaired motor development. The

hypoxic animals reared less at PND 25 (not at earlier age or older age) in the cylinder test, suggesting there was a sensorimotor developmental delay in the hypoxic animals. In the strength test, the hypoxic animals had shorter suspension times at PND 15, but had longer suspension times at PND 20. The shorter suspension times may have resulted from muscles weakness whereas the longer suspension times may have been due to a reduction in body weight. Although we did not weigh the animals, previous research has reported postnatal intermittent hypoxia can reduce body weight (Pozo et al. 2012). The results from suspension times indicate the hypoxic animals may have growth retardation. The morphologic alterations in motor cortex may explain the abnormal behavioral development after perinatal hypoxia (Hartle & Ivanco, 2010). In these behavioral tests, we also found as the animals matured, the behaviors became indistinguishable from control animals. This finding indicates neuroplasticity or recovery mechanisms may play roles in brain injury induced by perinatal hypoxia.

In the behavioral study, we did find brain developmental delay in some tests, including ambulating behavior, strength test, and cylinder exploration. The present study in rats may not be generalizable to humans. There are differences in brain development between humans and rats, for example, rat's brain is less mature than human's when they are born (Romijn et al., 1991). In humans, the motor and cognitive development is not delayed during the first 12 month after birth in the infants who are exposed to prenatal stress late in gestation (Davis & Sandman, 2010). In the study by Davis & Sandman (2010), only infants in early age were examined. These infants who had normal development may show abnormal behaviors at the old age. Our study in rats profiled a

relatively whole picture of spontaneous motor development from birth to young adult (PND 60).

The results of measuring stress hormones (Chapter 3) showed both corticosterone and cortisol were increased when the hypoxic rats were removed from the hypoxic chamber. The immediate increased concentrations of corticosterone and cortisol demonstrate hypoxia, as a stressor, activated the secretion of corticosterone and cortisol and the responses of corticosterone and cortisol to hypoxia were fast. Corticosterone and cortisol were found to have different expression patterns five days after hypoxia. The corticosterone level dropped down to the control level five days after removing from the hypoxic chamber, which may be due to high metabolism rate of corticosterone (as per Vazquez & Akil 1993; Romeo et al., 2004a,b, 2006a,b) or a fast response after removing from a stressor. On the contrary, the cortisol level was stable and high five days after hypoxia, suggesting cortisol may has lower metabolism rate (as per Gezici et al., 2009) compared to corticosterone or it may have its own functions (e.g. modulation of inflammation) after hypoxia. Although the cortisol level was consistently high five days after hypoxia, the concentration was much lower than the basal level of corticosterone, suggesting cortisol may not play a dominant role in laboratory rats. The basal level of corticosterone is more than 10 times higher than the basal level of cortisol, indicating corticosterone is the dominant stress hormone in laboratory rats, which is consistent with the literature (Xiong & Zhang, 2012). Although cortisol and corticosterone have similar effects, they are different molecules and may have different physiological functions (Vera et al. 2012). In the present study, the different concentrations and different expression patterns after hypoxia between corticosterone and cortisol support the above findings that

cortisol and corticosterone are different molecules and may play different roles during and after perinatal hypoxia.

We found cortisol and corticosterone had different response patterns after perinatal hypoxia. The present study in rats may not be fully parallel to humans. Different glucocorticoids are expressed between rats and humans. Corticosterone is the primary glucocorticoid in laboratory rats, but cortisol is the major glucocorticoid in humans (Xiong & Zhang, 2012). The cortisol levels in our study were stably high 5 days after the rats were taken out of the hypoxic chamber, indicating humans may have a longer responding time to hypoxia. The present study suggests prolonged hypoxia in the third trimester may cause the elevated levels of stress hormones in fetuses.

The study described in Chapter 4 showed altered innate and adaptive immune responses were found in brain tissue after perinatal hypoxia. These altered immune responses may be related to brain injury induced by perinatal hypoxia. The expression of three genes, *NF-κB*, *Tollip*, and *Traf6*, was up-regulated at PND 3 in the hypoxic group compared to the control group, indicating immune system may be activated at the early time after perinatal hypoxia. *NF-κB*, *Tollip*, and *Traf6* are not cytokine genes, but they may play a role in regulating the product of cytokines. Therefore, inflammation may not be obvious immediately after perinatal hypoxia. The gene expression of *Il1rl2, Il1rn, Pglyrp1, Ptafr, and TLR1* was up-regulated at PND 8 in the hypoxic group compared to the control group. In the hypoxic group, the gene expression of *Il1a, Ptafr, TLR6, TLR9, TNF-α,* and *Tollip* was up-regulated at PND 8 compared to PND 3, but the gene expression of *Adora2a* and *Cxcr4* was down-regulated. After the animals were taken out of the hypoxic chamber (PND 8), we found more genes, especially for cytokines, were

expressed compared to the early time after hypoxia exposure, suggesting immune responses may contribute to the secondary brain injury (as per Hedtjarn et al., 2004). One anti-inflammatory gene (*Il1rn*) was also found to be expressed five days after perinatal hypoxia, meaning recovery mechanisms may be also initiated. In addition, more genes related to modulation of immunity (immune modulators, e.g. *Pglyrp1*, *Ptafr*, *TLRs, Adora2a and Cxcr4*) were expressed, which may benefit or aggravate brain injury after perinatal hypoxia. Together, altered immune responses induced by perinatal hypoxia may play important roles mainly in the secondary brain damage and mechanisms related to recovery or anti-inflammation is activated, as well.

The components in inflammation (e.g. IL-1, IL-6, TNF, and IL-10) are usually considered biomarkers in brain injury (e.g. traumatic brain injury) (Woodcock & Morganti-Kossmann, 2013). In our study we found 14 genes related to innate and adaptive immunity were altered. These 14 genes may be potential biomarkers in perinatal hypoxia in rats. Future study should confirm the expression of these 14 genes by a larger sample size. In the study by Stridh et al. (2011), the authors did 5 replications from 5 different animals to confirm the gene expression that were significantly changed. The protein related to these genes that were significantly altered would be tested (e.g. western blotting) in the future research. These gene confirmation and protein tests would be next step to validate these 14 genes as biomarkers.

Studies have shown both stress hormones (Uno et al., 1990; Huang et al., 2001) and immune responses (Stridh et al., 2011; Hedtjarn et al., 2004; Kamel & Iadecola, 2012) can impair the developing brain and result in abnormal behavioral development (Wyrwoll & Holmes, 2012; Girard et al., 2012). In our study, we did not conduct

morphological examinations to assess brain injury, but the delayed behaviors were reflected by the extent of brain injury induced by perinatal hypoxia. Stress hormones and immune responses may be the biological basis of delayed behavioral development. Glucocorticoids are immune regulator and they can suppress immune responses after a chronic stress (Trinchieri et al., 1993; Monjan & Collector, 1977; Weiss et al., 1989; Dhabhar & McEwen, 1996). In the present study (refer to Chapter 3 and Chapter 4), the interaction between stress hormones and immune responses may determine the extent of brain injury. The high expression of corticosterone may suppress the immune response and result in the least gene expression of immune responses on PND 3 in the hypoxic animals. Normal levels of corticosterone may not suppress immune activity too much and notable alterations of gene expression of immune responses may occur on PND 8. The suppression of inflammation right after hypoxia exposure may be due to the high levels of glucocorticoids, which may be beneficial to the brain injury. However, high levels of glucocorticoids may impair the brain cells and structure (as per De Kloet et al., 1998; Fukumoto et al., 2009). During the recovery period, the glucocorticoids dropped down to the basal level and may not have harmful effects on brain. Meanwhile, the suppression of immune response was also reduced, which may result in the high expression of immune responses and may be associated with the secondary brain injury. Our data indicated an interaction between glucocorticoids and immune responses. We have provided Figure 15 as an overview of what might be occurring during this interaction. At PND 3, during the primary insult, the high level of corticosterone suppressed the immune responses, resulting in little gene expression of immune responses. At PND 8, during the secondary insult, the suppression of immune responses was declined due to the decreased level of

corticosterone, leading to significantly higher levels in gene expression of immune responses.

In the present study, the perinatal hypoxia model in rats caused the excess secretion of glucocorticoids and the activation of innate and adaptive immunity, which may mimick some features of extremely and/or very preterm birth. The 14 innate and adaptive immunity genes along with the increased levels of glucocorticoids are correlated with perinatal hypoxia in rats, also acting as potential biomarkers. The corticosterone levels were increased immediately following hypoxia at PND 3 and decreased at PND 8, which may be a biomarker in the primary insult of perinatal hypoxia, but also indicated the onset of a recovery process. The 11 genes of immune responses expressed 5 days after hypoxia may be biomarkers in the secondary insult. These potential biomarkers may also provide predictive mechanism for long term cognitive and learning disabilities in rats following perinatal hypoxia, which could be studied to determine generalizability to a human population. The detection of biomarkers may allow researchers to treat prenatal hypoxia at an early stage and give researchers some basic knowledge and the underlying mechanisms of extremely preterm birth, leading to better evidence based treatment options for the best long term outcome in children.

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Appendix

Setup ABI 7500 Fast System and Data Analysis of Real-Time RT-PCR

Preparation

The Rat Innate and Adaptive Immune Response RT^2 Profiler PCR Array were carried out using the ABI 7500 Fast System (Applied Biosystems, USA). A PCR protocol template file was set up on the ABI 7500 Fast System SDS software as follows:

1) Open the ABI 7500 Fast System SDS software on the desktop of the computer that is connected to the ABI 7500 system.
2) Select File ► New, the New Document Wizard dialog box appears.

   a) Select Assay ► Absolute Quantification (Standard Curve).

   b) Select Container ► 96-well Clear.

   c) Select Template ► Blank Document.

   d) Click Next, then select SYBR for the reporter dye-add it to the Detectors in Document box. Choose ROX for the Passive Reference box, then click Next.

   e) In the Set Up Sample Plate page, click the square button in the upper left corner of the diagram of the 96-well plate (between the letter "A" and "1") to select all wells. Once selected, the wells are highlighted in grey. Then check the "Use" box, next to SYBR, to indicate SYBR Green is used as the reporter dye for all wells.

   f) Click Fish, and then open the Plate document with the completed plate setup.

   g) Select the Instrument tab. Click to display the Thermal Profile box. In the Settings near the bottom of the screen, select Standard 7500 for Run Mode. A dialog box appears; click "Yes" when asked to reset the setting to the default

protocol. The Thermal Profile is concerted to the default settings of Standard 7500 run mode. Double click on the vertical line of the step you wish to delete; Step will turn black. Delete Stage 1 by clicking the Delete button. Then continue to edit the Thermal Profile as follows: Enter 95.0^{o}C for 10:00 minutes for Stage 1 with Reps 1; for Stage 2, enter 95.0^{o}C for 0:15 (15 seconds) followed by 60 ^{o}C for 1:00 minute with Reps 40 (40 cycles). Click the Add Dissociation Stage button; the pre-set dissociation stage is added as Stage 3. Put 25ul for Sample Volume and select Stage 2, Step 2 (60.0 @1:00) for Data Collection.

h) Then select File ► Save As to save the template file. Save the file as SDS Templates (*.sdt) with the filename "RT^{2} Profiler^{TM} PCR Array Protocol Template" (Click Save).

Performing Real-Time PCR Detection

The ABI 7500 Fast System and the computer connected to the thermocycler were switched on. Opened the tray and placed the plate in the precision plate holder with the last row (row H) facing front. Made sure the plate was properly aligned in the holder, well A1 was positioned at the top-left corner of the tray and closed the tray door. The ABI 7500 Fast System SDS Software was open and the experimenter loaded the RT^{2} Profiler^{TM} PCR Array Template as following: Selected File ► New, in the New Document Wizard dialog box, selected Browse to load the Template file and then clicked finished. This loaded the previously saved setup to the new plate document, and saved the new document under a new filename as SDS Document (*sds). Selected the Instrument tab, click Start to begin the PCR run. Waited 30 seconds to 1 minute for the initial priming, then the run started, and the estimated run time appeared on the screen.

After the PCR run

When the PCR run was complete, a small dialog box stating "The run completed successfully" appeared on the screen. Clicked OK, and then closed the box. Clicked the Result tab and choose the Amplification Plot page. Display Data as Delta Rn vs Cycle, clicked the square button in the upper left corner of the diagram of the 96-well plate (between the letter "A" and "1") to select all wells. The selected wells were highlighted in grey. Procedures below were followed to calculate the threshold cycle (Ct) for each well.

a) To define the baseline, use the Linear View of the amplification plots. Selected Tool ► Graph Settings. The window for Graph Settings appeared. For Post-Run Settings, selected Auto Scale for both the Y and X-axes. Selected Linear view for Y-Axis, then clicked OK. With the linear plots, determine the cycle number at which the earlieat amplification can be seen. Selected Analysis ► Analysis Settings. Set the Manual Baseline to start from cycle number 2 through two cycle values before the earliest visible amplification, then clicked OK.

b) To define the Threshold Value, used the Log View of the amplification plots. Selected Tool ► Graph Settings. In the Post-Run Settings, selected Log view for the Y-Axis, then clicked OK. With the log plots, placed the threshold line above the background signal but within the lower third of the linear phase of the amplification plot. The threshold value was set as 0.05 in our study. Then selected Analysis ► Analyze.

c) Went to the Report page to display the value for Ct and the melting temperature (Tm) for each well. To explore the result to an Excel spreadsheet, selected File ► Export ► Results and save the file as Results Export Files (*csv).

Data analysis of Real-Time RT-PCR using the ΔΔCt method

The online data analysis software uses ΔΔCt method. To get ΔΔCt, ΔCt should be calculated first. Calculate the ΔCt for each pathway-focused gene in each plate using the Ct values for the gene of interest (GOI) and the housekeeping genes used for normalization (HKG) and the formula is

$$\Delta Ct = Ct^{GOI} - Ct^{AVG\ HKG}.$$

The formula used to calculate ΔΔCt is that

$$\Delta\Delta Ct = \Delta Ct\ (group2) - \Delta Ct(group1)$$

Group 1 is the control sample or groups of samples and group 2 is the experimental sample or group of experimental samples.

Data are reported as fold change relative to control. Fold change is calculated as below. Due to the inverse proportional relationship between the threshold cycle (Ct) and the original gene expression level, and the doubling of the amount of product with every cycle, the original expression level (L) for each gene of interest is expressed as:

$$L = 2^{-Ct}$$

To normalize the expression level of a gene of interest (GOI) to a housekeeping gene (HKG), the expression levels of the two genes are divided:

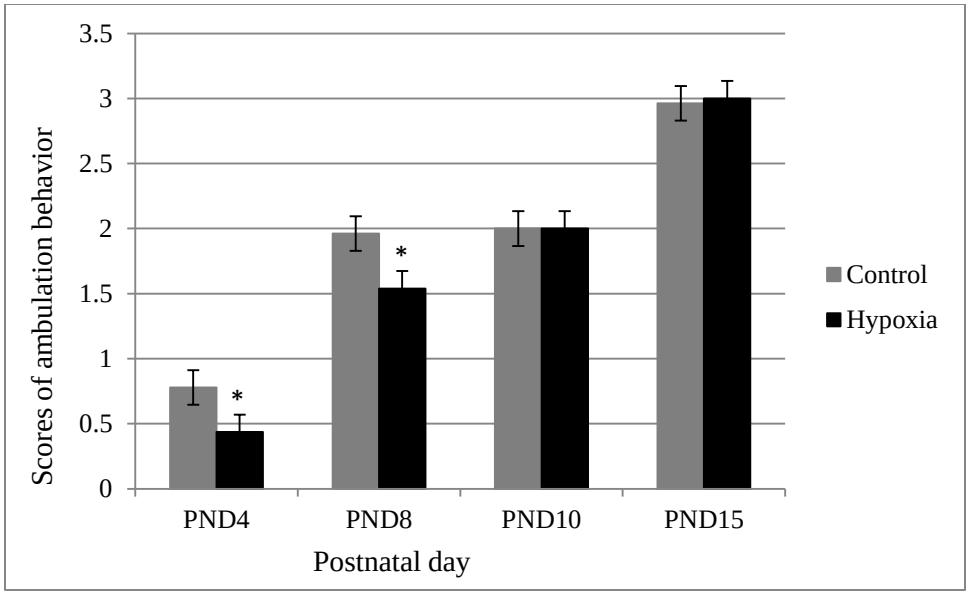
$$\frac{2^{-C_t(GOI)}}{2^{-C_t(HKG)}} = 2^{-[C_t(GOI) - C_t(HKG)]} = 2^{-\Delta C_t}$$

To determine fold change in gene expression, the normalized expression of the GOI in the experimental sample is divided by the normalized expression of the same GOI in the control sample. The complete calculation is as follows:

$$\frac{\frac{2^{-\Delta C_t(GOI)}\,expt}{2^{-\Delta C_t(HKG)}\,expt}}{\frac{2^{-\Delta C_t(GOI)}\,control}{2^{-\Delta C_t(HKG)}\,control}} = \frac{2^{-[C_t(GOI)\,-\,C_t(HKG)]}\,expt}{2^{-[C_t(GOI)\,-\,C_t(HKG)]}\,control} = \frac{2^{-\Delta C_t}\,expt}{2^{-\Delta C_t}\,control} = 2^{-\Delta\Delta C_t}$$

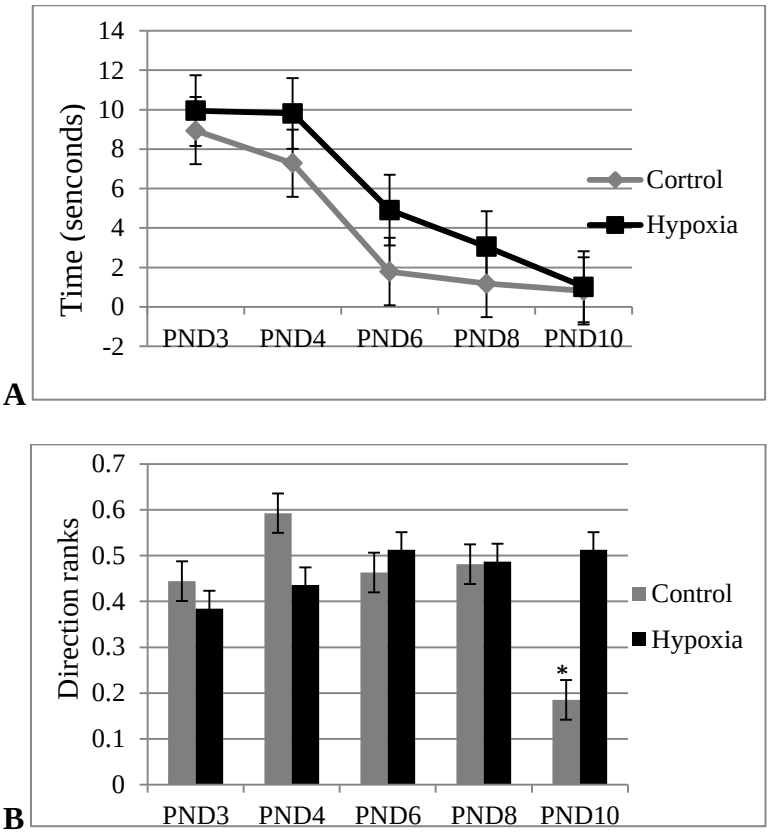
Fold change, experiment relative to control, is obtained. A student's *t* test is used to evaluate whether fold change is significantly different to control.

Figure 1: Ambulating behavior



Mean scores (± SEM) of ambulating behavior were obtained in each trial as a function of experimental condition. The x-axis is the age at which the animals were tested and the y-axis is the median scores obtained in the trials. The ambulating scores in hypoxic animals were significantly lower than control animals on PND 4 and PND 8. There were no significant differences between the two groups on PND 10 and PND 15. (* = $p < 0.05$ vs control).

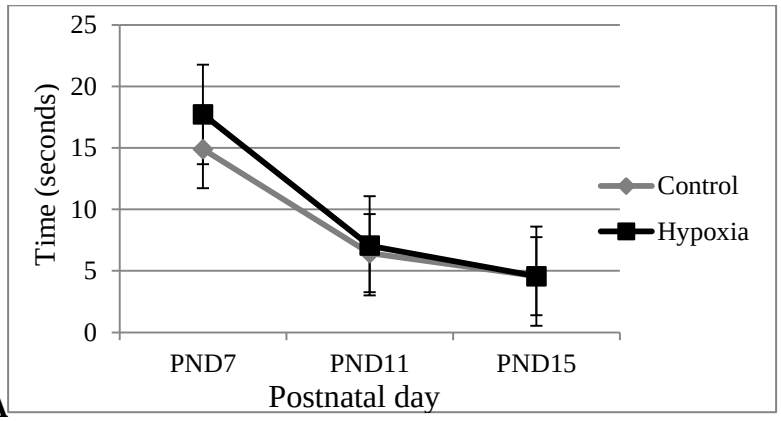
Figure2: Righting behavior



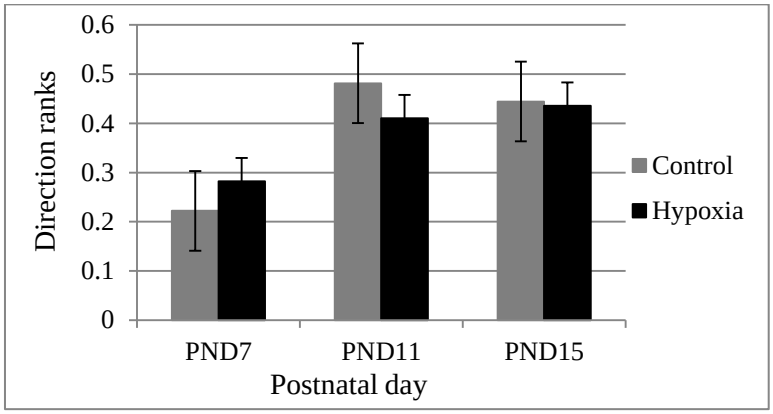
A. Mean number (±SEM) seconds taken to complete the task as a function of experimental condition. The x-axis is the age at which the animals were tested and the y-axis is the average time taken to complete the task. There was no significant difference in the time required to complete the task across days between hypoxic and control animals. A significant condition of days revealed a decrease in the time the animals were taken to complete the task across days.

B. Mean ranks (± SEM) of direction that the animals were turned from supine to quadruped position as a function of experimental condition. There was a significant difference of direction on PND 10 between the two groups. No significant differences were found on PND 3, PND 4, PND 6, and PND 8. (* = $p < 0.05$ vs control).

Figure 3: Negative geotaxis



A

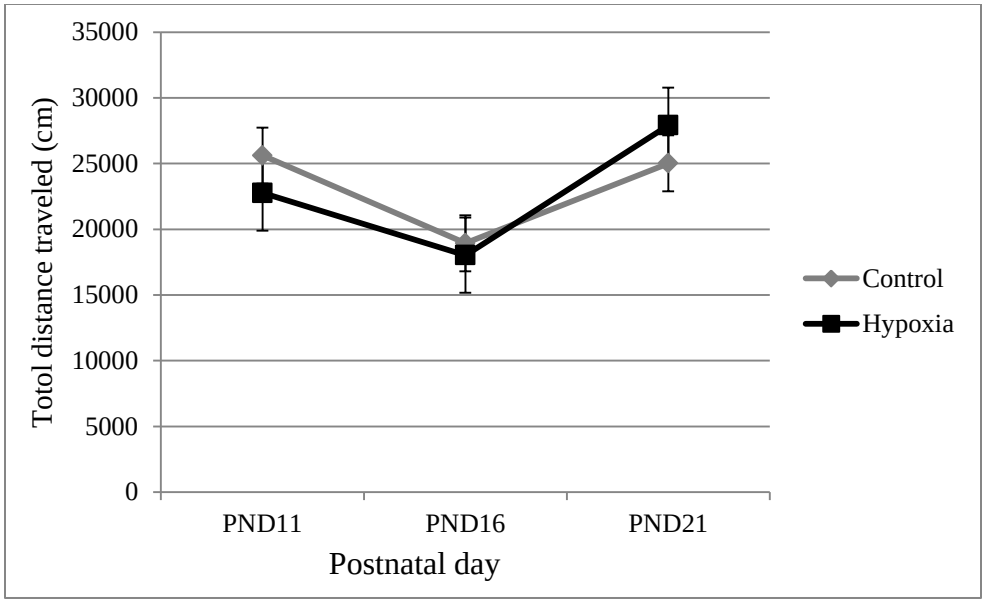


B

A. Mean number (±SEM) of seconds the animals were required to face the inclined position. The x-axis is the age at which the animals were tested and y-axis is the average time the animals were taken to face the inclined position. The only significant finding was a decrease in the amount of time the animals were able to face the incline position across days. There was no significant difference in the time required to face the inclined position between experimental and control animals.

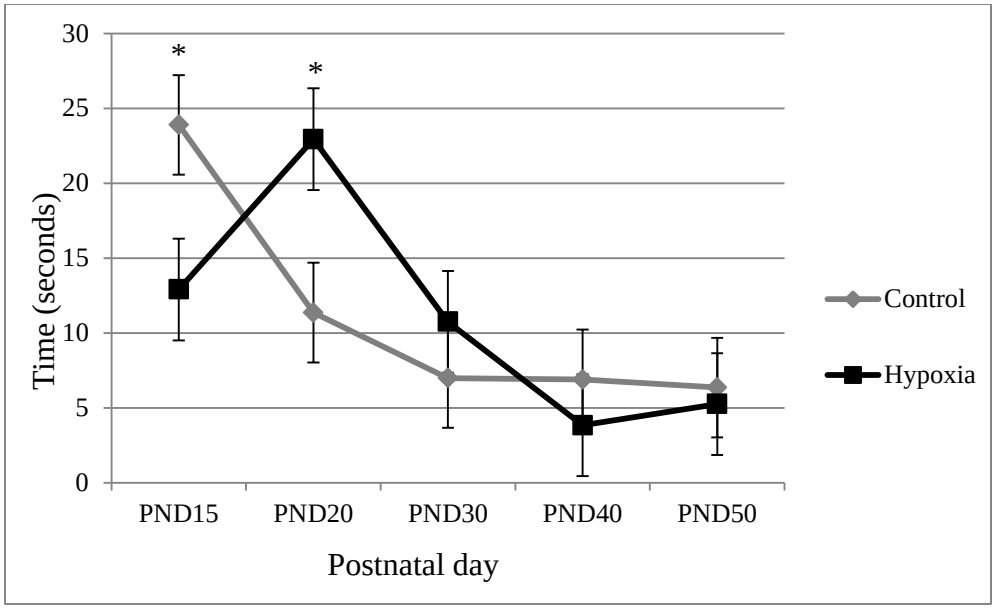
B. Mean ranks (± SEM) of direction that the animals turned from the declined position to the inclined position as a function of experimental condition. There was no significant difference of direction at different time points between experimental and control animals.

Figure 4: Explorative behavior



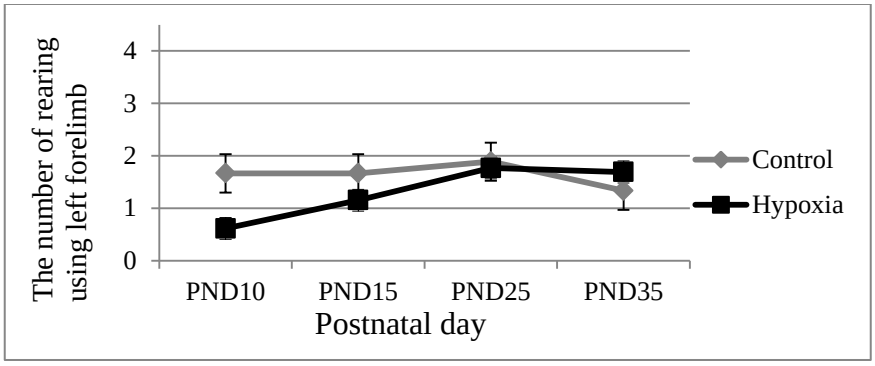
Mean number (±SEM) of centimeters traveled within 20 minutes in an open field box. The x-axis is the age at which the animals were tested and the y-axis is the total distance traveled. No significant difference was found in the total distance traveled within 20 minutes in an open field box across days between hypoxic and control animals. There was a significant days X condition interaction due to a decrease of distance traveled on PND 16 in both groups of animals.

Figure 5: Strength test

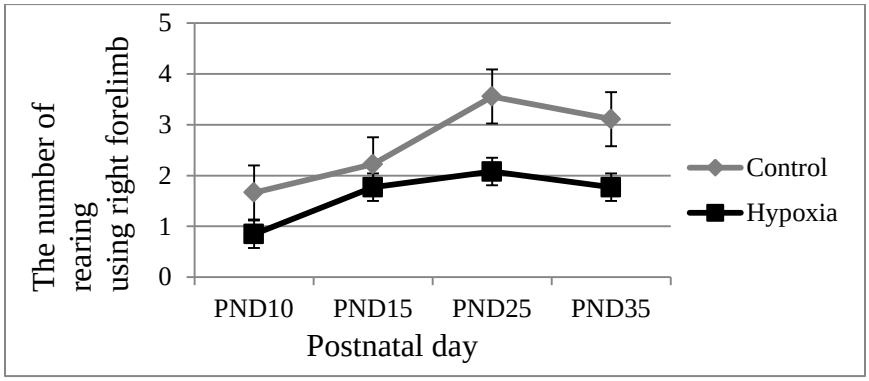


Mean number (±SEM) seconds the animal was able to hang from the dowel. The x-axis is the ages at which the animals were tested and the y-axis is the average length of time the animal was able to stay suspended. Hypoxic animals stayed suspended with less length of time than control animals on PND 15, but hypoxia animals stayed suspended with more length of time than control animals on PND 20. There is also a significant effect of days indicating that the length of time the animals was able to hang on to the dowel was changed across days. (** = $p < 0.01$ vs control).

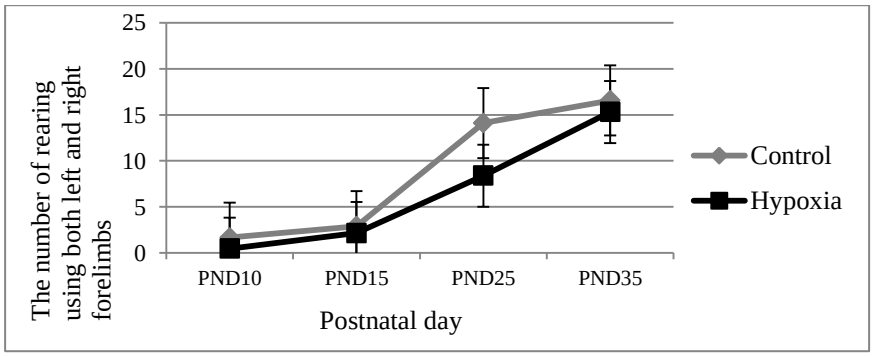
Figure 6: Cylinder exploration



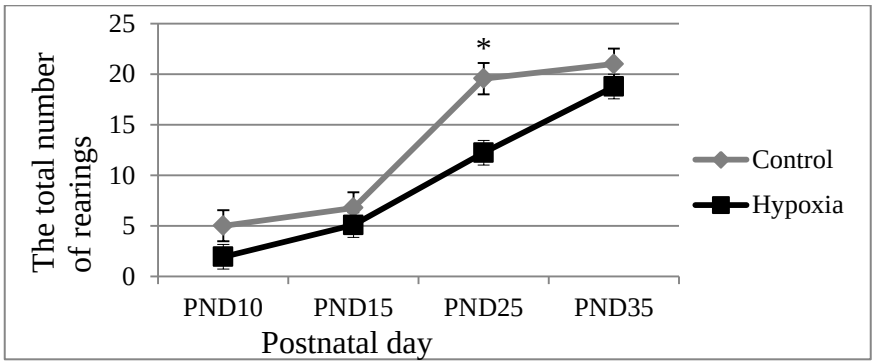
A



B



C



D

A. Mean number (±SEM) of rearings the animal made with left paws. The x-axis is the ages at which the animals were tested and the y-axis is the average number of rearings the animal made with left paws. No significant difference was found in the number of rearings animal made with left paws across days between hypoxic animals and control animals.

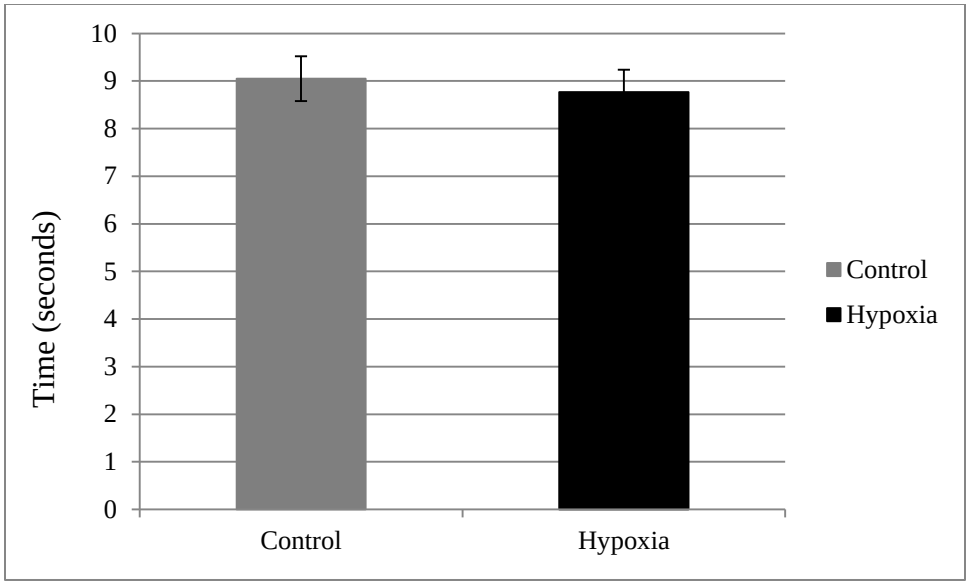
B. Mean number (±SEM) of rearings the animal made with right paws. The x-axis is the ages at which the animals were tested and the y-axis is the average number of rearings the animals made with right paws. No significant difference was found in the number of rearings animal made with right paws across days between experimental animals and control animals.

C. Mean number (±SEM) of rearings the animal made with both paws. The x-axis is the ages at which the animals were tested and the y-axis is the average number of rearings the animals made with both paws. No significant difference was found in the number of rearings animal made with both paws across days between hypoxic animals and control animals. There was a significant effect of days indicating an increase of usage of both forepaws to explore across days regardless of conditions.

D. Mean number (±SEM) of total number of rearings animal made during the testing time. Total number of reearings means all rearings were added together (D = A + B + C). The x-axis is the ages at which the animals were tested and the y-axis is the average number of total number of rearing animal made. There is a significant effect of conditions and it was found that experimental animals at PND 25 did less rearings than control

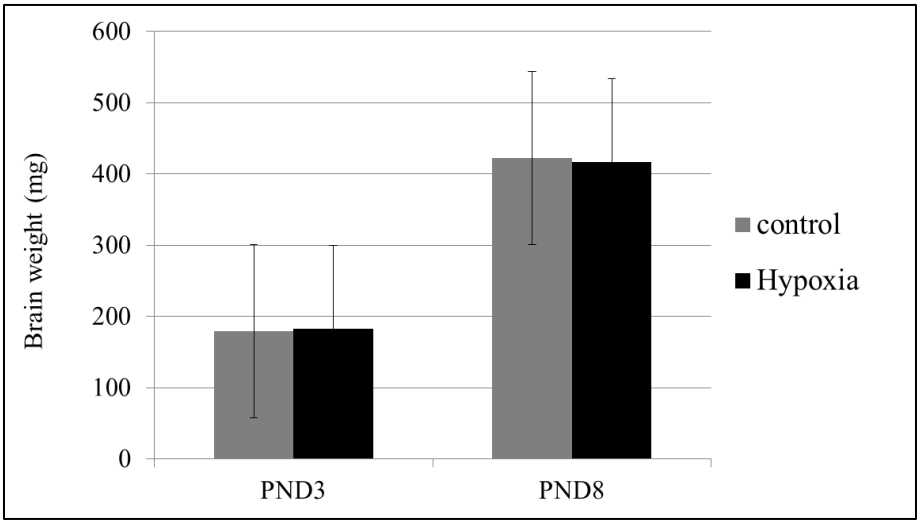
animals did. A significant increase of the total number of rearings the animals made across days was found in this test. (* = $p < 0.05$ vs control).

Figure 7: Ladder runway



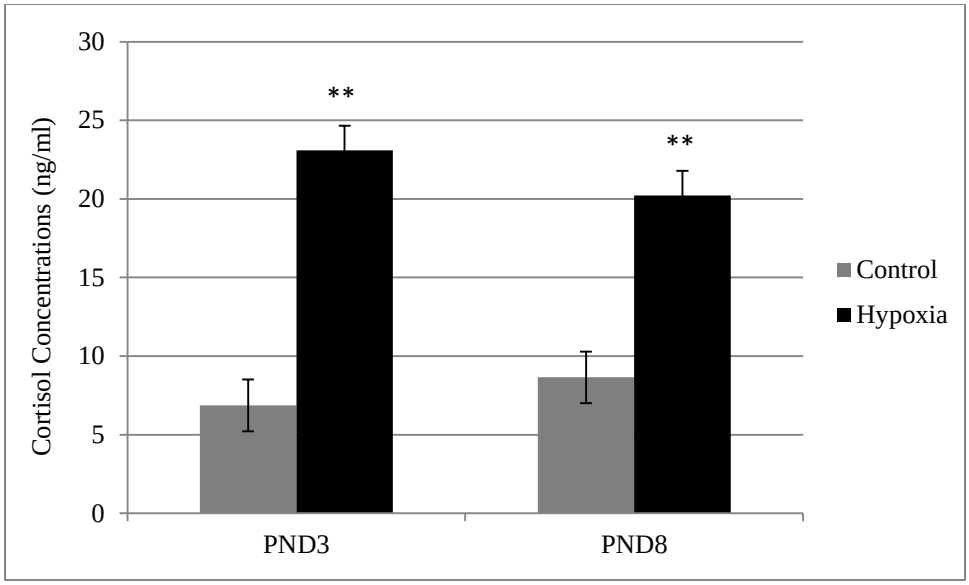
Mean number (±SEM) of time in seconds taken to traverse a runway. The x-axis is conditions and y-axis is the average time taken to traverse a ladder runway. There was no significant difference in the time animals took to traverse the runway.

Figure 8. Brain weight



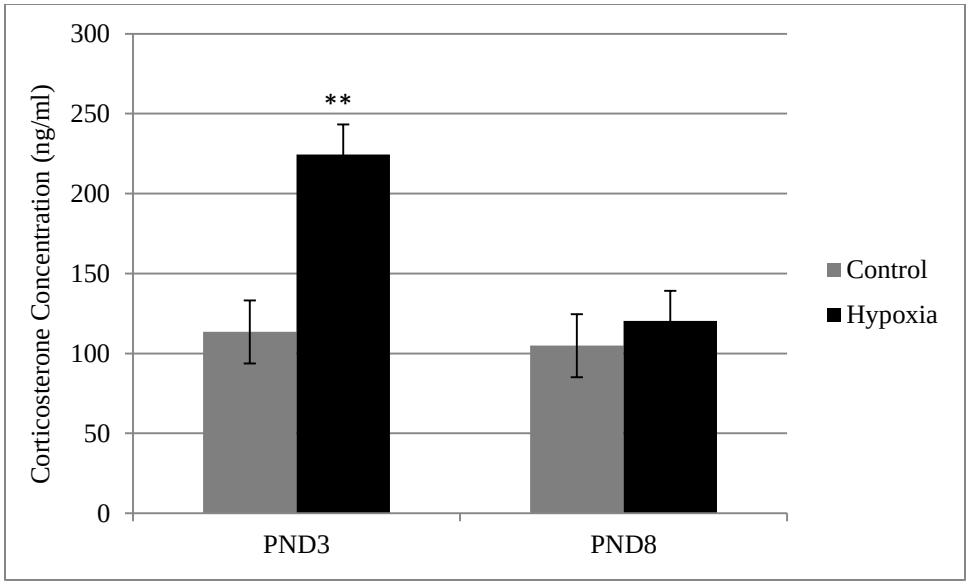
Mean number (±SEM) of brain weight. The x-axis is the ages at which the brain weight was measured and the y-axis is the average micrograms of the brain weight. There was no significant difference in the brain weight between groups at both PND 3 and PND 8. A significant increase of brain weight across days was found in this test.

Figure 9: Different cortisol concentrations from the hypoxic and the control groups



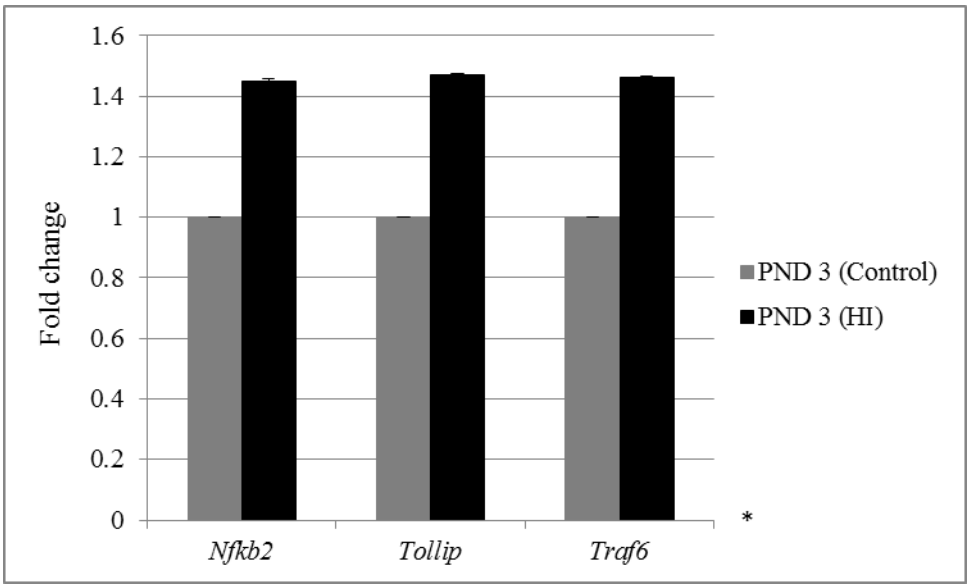
Mean cortisol concentrations (±SEM) at PND 3 and PND 8 in the hypoxic animals and the control animals. The x-axis is the ages at which the animals were tested and y-axis is the mean concentration of cortisol. It was found that cortisol concentrations were significantly increased at both PND 3 and PND 8 in the hypoxic group compared to the control group (** = $p < 0.01$ vs control).

Figure 10: Different corticosterone concentrations from hypoxic and control groups



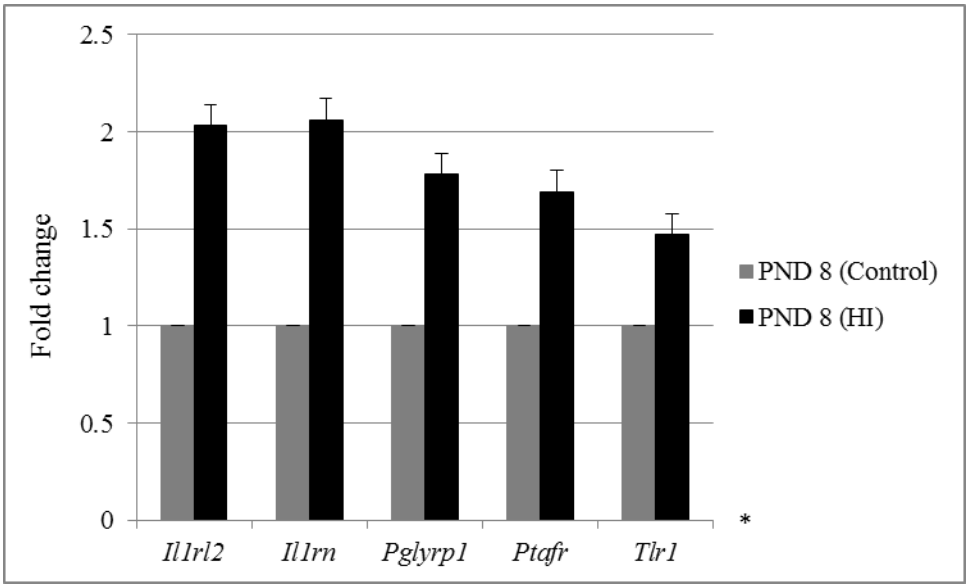
Mean corticosterone concentrations (±SEM) at PND 3 and PND 8 in the hypoxic animals and the control animals. The x-axis is the ages at which the animals were tested and y-axis is the mean concentration of corticosterone. Corticosterone concentrations were significantly increased at PND 3 in the hypoxic animals compared to the control animals. The corticosterone concentrations were significantly higher at PND 3 than at PND 8 in the hypoxic animals. (** = $p < 0.01$ vs control).

Figure 11: Fold change values of significantly differently expressed genes at PND 3 in hypoxic group compared to control group.



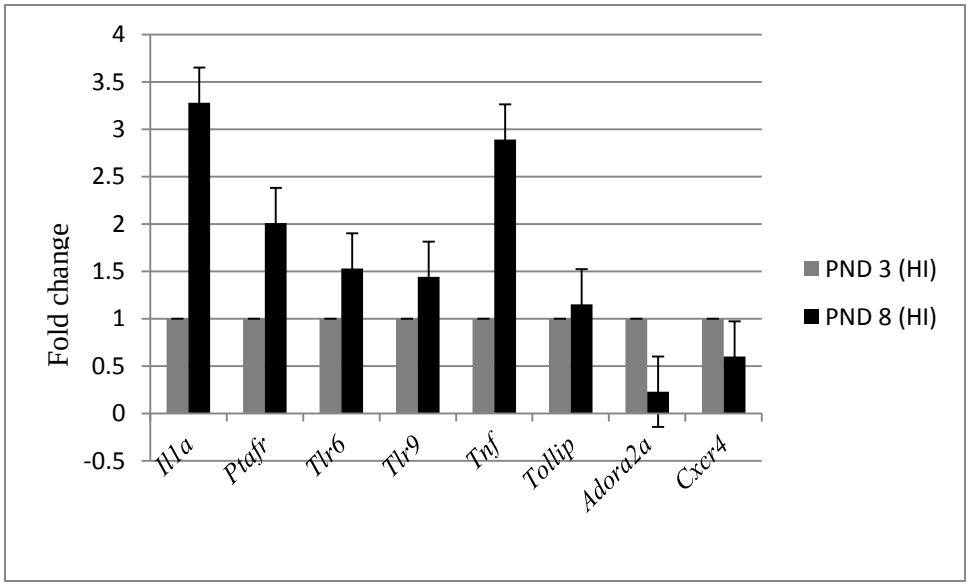
The genes expression of immune responses at PND 3 in the hypoxic animals was compared with the genes expression of immune responses at PND 3 in the control animals. Only significant changes of fold changes are reported. Greater than 1 means up-regulation.

Figure 12: Fold change values of significantly differently expressed genes at PND 8in hypoxic group compared to control group.



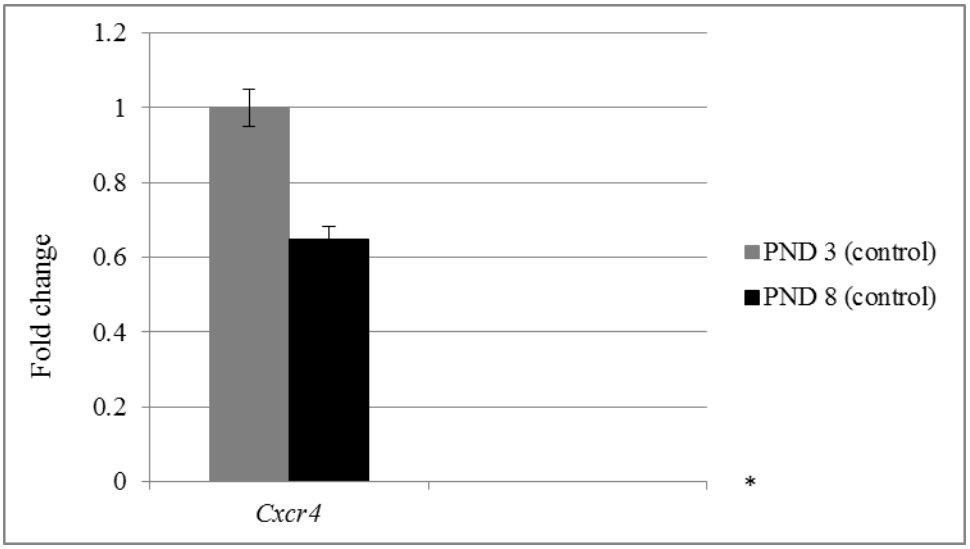
The genes expression of immune responses at PND 8 in the hypoxic animals was compared with the genes expression of immune responses at PND 8 in the control animals. Only significant changes of fold changes are reported. Greater than 1 means up-regulation.

Figure 13: Fold change values of significantly differently expressed genes at PND 8 compared to PND3 in hypoxic animals.



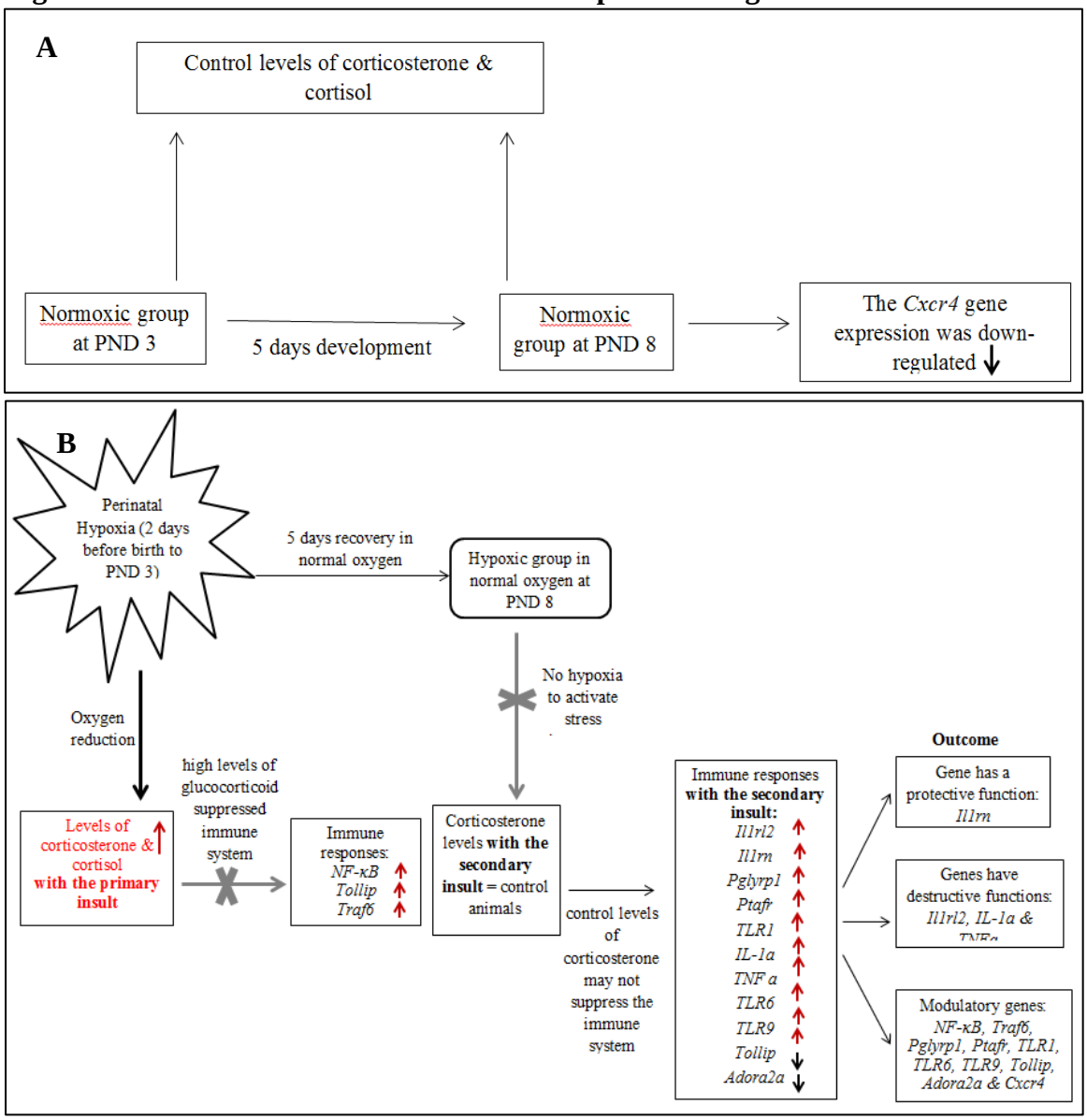
The genes expression of immune responses at PND 8 was compared with the genes expression of immune responses at PND 3 in the hypoxic animals. Only significant changes of fold changes are reported. Greater than 1 means up-regulation and less than 1 means down-regulation.

Figure 14: Fold change values of significantly differently expressed genes at PND 8 compared to PND 3 in control animals.



The genes expression of immune responses at PND 8 was compared with the genes expression of immune responses at PND 3 in the control animals. Only significant changes of fold changes are reported. Less than 1 means down-regulation.

Figure 15. The intraction between immune responses and glucocorticoids



A: Model picture in the normoxic group.

B: Model picture in the hypoxic group. The suppression of inflammation at PND 3 may be due to the high levels of glucocorticoids during the primary insult. At PND 8, the secondary insult, the glucocorticoids dropped down to the basal level and the suppression of immune response was reduced, resulting in the high expression of immune responses.

Table 1: Cortisol standard preparation

	Dilution	Final Conc. (pg/mL)
Std. 1	Standard (50ul) + Assay Buffer (450ul)	3,200
Std. 2	Std. 1 (250ul) + Assay Buffer (250ul)	1,600
Std. 3	Std. 2 (250ul) + Assay Buffer (250ul)	800
Std. 4	Std. 3 (250ul) + Assay Buffer (250ul)	400
Std. 5	Std. 4 (250ul) + Assay Buffer (250ul)	200
Std. 6	Std. 5 (250ul) + Assay Buffer (250ul)	100

This table described how to prepare cortisol standard. Assay buffer (450ul) was added into tube #1, and 250ul was added into tube #2 to #6. Supplied standard cortisol (50ul) was added into tube #1. Cortisol solution (250ul) in tube #1 was taken out and added it to tube #2, and the serial dilutions were repeated for tube #3 through #6. The concentration of standard cortisol in tubes 1 through 6 was 3200, 1600, 800, 400, 200, and 100pg/mL.

Table 2 (Corticosterone standard preparation)

	Dilution	Final Conc. (ng/mL)
Std. 1	Standard (100ng 80 µl)	100.00
Std. 2	Std. 1 (20µl) + EIA Diluent (60µl)	25.00
Std. 3	Std. 2 (20µl) + EIA Diluent (60µl)	6.250
Std. 4	Std. 3 (20µl) + EIA Diluent (60µl)	1.563
Std. 5	Std. 4 (20µl) + EIA Diluent (60µl)	0.391
Std. 6	EIA Diluent (80 µl)	0.000

This table described how to prepare corticosterone standard. The standard solution (100ng/mL 80µl) was added into the tube #1, and EIA Diluent (60µl) was added into tube #2 to tube #5. The standard solution (20µl) was taken out from tube#1 and added into tube #2, and the serial dilutions 1:4 were repeated for tube #3 through #5. EIA Diluent 80µl was added into the tube #6. The concentration of standard corticosterone in tubes 1 through 6 was 100ng/mL, 25ng/mL, 6.25ng/mL, 1.563ng/mL, 0.391ng/mL, and 0ng/mL.