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LOMA LINDA UNIVERSITY
School of Medicine
in conjunction with the
Faculty of Graduate Studies

Effect of Maternal Hypoxia on Fetal Development: Programming of
HIE Phenotype in Neonatal Rat Brain

by

Pablo J González-Rodríguez

A Dissertation submitted in partial satisfaction of
the requirements for the degree of
Doctor of Philosophy in Biochemistry

June 2014

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

3M	Three month
11b-HSD2	11-Beta hydroxysteroid dehydrogenase isozyme 2
ACE	Angiotensin converting enzyme
Ang-2	Angiopoietin-2
Ang II	Angiotensin II
ANOVA	Analysis of variance
AT ₁ R	Angiotensin type-1 receptor
AT _{1a} R	Angiotensin type-1a receptor
AT _{1b} R	Angiotensin type-1b receptor
AT ₂ R	Angiotensin type-2 receptor
Bax	Bcl-2-like protein 4
BBB	Blood-brain barrier
Bcl-2	B-cell lymphoma 2
BDNF	Brain-derived neurotrophic factor
BPA	Bisphenol A
CA1	Cornu ammonis 1
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CB1	Cannabinoid receptor type 1
ChIP	Chromatin immunoprecipitation
CKD	Chronic kidney diseases
CNS	Central nervous system

COX	Cyclooxygenase
CRTH2	Chemoattractant receptor homologous expressed on Th2 cells
DADLE	[d-Ala2, d-Leu5] enkephalin
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
DNMT	DNA methyl transferase
DOR	Delta opioid receptor
DP1,2	Prostaglandin D2 receptor type 1 or 2
E21	Embryonic day twenty-one
Egr-1	Early growth response protein 1
ERK 1/2	Extracellular signal-regulated kinase 1,2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
Gai	Receptor activated G protein alpha inhibitory
Gas	Receptor activated G protein alpha stimulatory
GDNF	Glial cell-derived neurotrophic factor
GFR	Glomerular filtration rate
GR	Glucocorticoid receptor
GSH	Glutathione reduced state
GSSG	Glutathione oxidized state
H3K9	Histone h3 lysine 9
H3K14	Histone h3 lysine 14
HIE	Hypoxic-ischemic encephalopathy
HIF-1 α	hypoxia-inducible factor 1-alpha

HPA	Hypothalamic-pituitary-adrenal axis
HPTM	Histone posttranslational modification
ICV	Intracerebroventricular injection
IP	Intraperitoneal injection
IUGR	Intrauterine growth restriction
LIFE	Losartan intervention for endpoint
LPGDS	Lipocalin-type prostaglandin D synthase
LXR α	Liver X receptor alpha
MAPK	Mitogen-activated protein kinase
MBD1	Methyl-CpG-binding domain protein 1
MCAO	Middle cerebral artery occlusion
MDD	Methyl donor diet
MeCP2	Methyl CpG binding protein 2
MeDIP	Methylated DNA Immunoprecipitation
MMP-2,3,9	Matrix metalloproteinases 2,3 or 9
MOSES	Morbidity and mortality after stroke: eprosartan versus nitrendipine in secondary prevention
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
NGFI-A	EGR-1-binding protein 2
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NTI	Naltrindole
P1,5,7,10	Post-natal day 1,5,7 or 10

p53	Protein 53
pdx1	Pancreatic and duodenal homeobox 1
pERK	Phosphorylated extracellular signal-regulated kinase
PGD2	Prostaglandin D2
PGH2	Prostaglandin H2
PKC ϵ	Protein kinase C epsilon
RAS	Renin-angiotensin system
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SEM	Standard error of mean
sGC	Synthetic glucocorticoids
Sp1	Specific protein 1
StAR	Steroidogenic acute regulatory protein
TBST	Tris buffered saline with tween
TNF- α	Tumor necrosis factor alpha
TTC	Tetrazolium chloride
USF1	Upstream transcription factor 1
Wnt4,11	Wingless-type MMTV integration site family, member 4 or 11

ABSTRACT OF THE DISSERTATION

Effect of Maternal Hypoxia on Fetal Development: Programming of HIE Phenotype in Neonatal Rat Brain

by

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Doctor of Philosophy, Graduate Program in Biochemistry
Loma Linda University, June 2014
Dr. Lubo Zhang, Chairperson

Developmental programming of health and disease correlates the adverse intrauterine environment and the risk of disease later in life as cardiovascular disease, metabolic diseases, renal dysfunction or neurobehavioral disorders among others. Maternal hypoxia is one of the most common intrauterine growth restricted environment during pregnancy. Perinatal hypoxic-ischemic encephalopathy is an important cause of brain injury in the newborn due to a systematic asphyxia. Our study aims to test the hypothesis that maternal hypoxia affect the organogenesis of the kidney by affecting the kidney structure and focusing on the roles of AT₁R and AT₂R, while the brain development will also be affected by this maternal insult increasing the vulnerability of this to HIE brain injury and the role of GR and the epigenetic modification that regulate the expression of the GR will be assessed. Finally, the MAPK/ERK pathway as part of the downstream of the neuroprotective mechanism of the GR against neonate HIE brain injury will be evaluated. To address these issues two rats model were established: perinatal hypoxia exposure model in time-dated pregnant rats and hypoxic-ischemic encephalopathy in 10-day-old rat pups. Through these studies we found that maternal hypoxia decrease the number of glomeruli in offspring rat kidneys and a down-regulation on the expression of AT₁R was observed in the kidneys of the adult rats in a sex

dimorphism way, affecting more the females than the males. In addition, we observed that maternal hypoxia increased the vulnerability of the brain to HIE on P-10 pups by repressing the expression of GR. Epigenetics studies demonstrated that this down-regulation of GR was due to hypermethylation on promoter region of exon variants 17 and 111 of the GR gene. Dexamethasone, a synthetic glucocorticoid, treatment on normoxic P-10 brains increased the expression of LPGDS, and pERK44 and also the level of prostaglandin D2, while, the neuroprotective effect of dexamethasone observed against HIE brain injury on P-10 pups was diminished by MAPK/ERK and LPGDS inhibitor, and by blocking the DP1 receptor. These findings provide a new insight in understanding the pathogenesis of HIE in newborns and may suggest potential targets for the prevention and treatment of HIE brain injury.

CHAPTER ONE

INTRODUCTION

Programing Diseases

Adverse fetal environment, mostly manifested as intrauterine growth restriction (IUGR), contribute to affect organogenesis and furthermore, may affect organ function at maturity increasing the probability for an individual to have a variety of health problems such as coronary heart disease, type 2 diabetes, neurobehavioral disorders, hypertension, cardiovascular disease, or renal dysfunction among others (Dudley, Li, Kobor, Kippin, & Bredy, 2011; Harris & Seckl, 2011). This adverse insult during fetal development may lead to low birth weight, which is a common marker of IUGR in the offspring. The hypothesis of “developmental programming of health and disease” or “fetal origins of adult disease” was put forward to elucidate these links between adverse intrauterine environment, fetal growth and development, and disease later in life.

Environmental signals can be transmitted from the mother to the fetus, impacting specific vulnerable tissues in their sensitive developmental stage, modulating normal development trajectory, remodeling their structure and function and reprogramming the resiliency or susceptibility to diseases in postnatal life.

Plenty of studies, using different maternal perturbation, have demonstrated a significant effect on the organogenesis due to those adverse environments. Malnutrition is one of the most common that may contribute to perinatal programming, and both ends have been extensively studied, from undernutrition (caloric and protein restriction diets or

low-vitamin intake) (Erkkola, Nwaru, & Viljakainen, 2011; Mackay, Khazall, Patterson, Wellman, & Abizaid, 2013; Woods, Ingelfinger, Nyengaard, & Rasch, 2001) to overnutrition (high-fat, high-energy and high-protein diets) (Elahi et al., 2009; Gniuli et al., 2008; Oster, Murani, Metges, Ponsuksili, & Wimmers, 2012). Another environmental stress for fetal development is maternal smoking. It is estimated that approximately 22% of pregnant mothers smoke during the gestational period (Nelson & Taylor, 2001). The use of nicotine during pregnancy has been reported to affect the organogenesis of the brain (Bruin, Gerstein, & Holloway, 2010) and the heart (Xiao, Huang, Yang, & Zhang, 2011) and eventually it affects the function of these organs during adulthood. Brain and cardiovascular programming diseases also occurred when the fetus is exposed to cocaine (Gressens, Mesples, Sahir, Marret, & Sola, 2001; Xiao, Huang, Xu, Yang, & Zhang, 2009). Plenty of evidence has demonstrated that fetal stress induces an abnormal development of organs and could program them to be more vulnerable to some kind of disease or condition later in life.

Kidney Development

The “developmental programming of health and disease” hypothesis has been extended to other adult diseases as lung function, (Stein et al., 1997) learning ability (Strang-Karlsson et al., 2008), immune function (McDade, Beck, Kuzawa, & Adair, 2001) and also renal function (Manalich, Reyes, Herrera, Melendi, & Fundora, 2000). One of the most important functions of the kidney is to filtrate urine by small structures called nephrons. A human kidney contains an average ~ 1 million nephrons, but the range in normal human population varies considerably (Hughson, Douglas-Denton, Bertram, & Hoy, 2006). Hypertension is one of the most common diseases associated with fetal

programming. It has been hypothesized that high risk for hypertension is due to a congenital deficiency in the number of nephrons, which results in a reduction of the filtration surface area that programs a subsequent reduction in glomerular filtration rate. This reduction in GFR will lead to an increase in sodium retention and eventually hypertension (Mackenzie & Brenner, 1995). Human and animal studies of low birth weight newborns demonstrate a reduced glomerular number. Thus, the process and consequence of reduced intrauterine growth is sufficient to impair renal development. Furthermore, a reduction in the nephron number and an increase blood pressure on the offspring has been reported in studies using different maternal adverse environment such as low protein diet (Woods et al., 2001), glucocorticoids (Ortiz, Quan, Zarzar, Weinberg, & Baum, 2003), placenta insufficiency (Wlodek, Westcott, Siebel, Owens, & Moritz, 2008) and maternal sodium overload (Koleganova et al., 2011).

In rats, the second half of gestation is a critical period for renal development because nephrogenesis process is during this time; being the late renal development period, the most susceptible due to the nephron number increasing exponentially at this time. Therefore, any kind of fetal adverse environment during nephrogenesis will induce an impairment renal development that cannot be restored later in gestation, even if the adverse environment is removed (O'Sullivan et al., 2013; Singh et al., 2007).

The renin angiotensin system has been associated in certain of these program outcomes. It is known that RAS is often repressed during renal development, potentially contributing to the nephron deficit, followed by a compensatory increase in activity in postnatal life (Moritz et al., 2010). Given the essential function of the RAS in fluid balance and blood pressure control in the adult, long-term up-regulation of this system,

combined with the low nephron endowment, provides a highly plausible pathway through which prenatal insults may result in hypertension.

Brain Development

The brain is one of the critical targets of stressors and is also the central organ responsible for stress responses, determining the adaptive or maladaptive responsiveness to various acute and chronic stressful events via making corresponding alterations in its structure and function (McEwen, 2008). The developing brain in the fetal stage is also highly plastic, flexible, and especially sensitive to numerous adverse environmental factors. Combined with its specific genetic traits, these changes of fetal brain contribute to a high incidence of a wide spectrum of neurodevelopmental disorders in the postnatal life. It has been well documented that fetal stresses, such as hypoxia, malnutrition, substances exposure (nicotine, alcohol and cocaine) and excess glucocorticoids (endogenous or exogenous), have long lasting impact on the developing brain; altering brain's ontogeny, organization, structure and function; remodeling brain's development trajectory, and reprogramming brain's vulnerability or resiliency of some neurobehavioral, neuropsychological and neuropsychiatric disorders later in life (Harris & Seckl, 2011).

It is known that the last week of gestation for mice and rats, where fetal brain development is taking place, is parallel with the first-second trimester of human fetal development and the development of the rat brain during the first two postnatal weeks are parallel with the third trimester in human fetal brain (Golan & Huleihel, 2006). Animal studies have provided evidence linking maternal adverse environment and the increased incidence of neurodevelopmental disorders, neurobehavioral deficits, impaired cognitive

performance, and increased risk of affective disorders later in life (Y. Li et al., 2012; Maier, Chen, Miller, & West, 1997).

Maternal Hypoxia

Another common hostile environment associated with IUGR is maternal hypoxia, which is a reduced level of oxygen during pregnancy; this is one of the most significant challenges facing obstetric clinic practice. There are various possible scenarios, which could induce a hypoxia environment to the fetus. Fetal hypoxia is very common in pregnant women that live at high altitude; also, unfortunately, many pregnant women continue to smoke during pregnancy, as was mentioned before, exposing the fetus to prolonged hypoxemia. Furthermore, the placenta plays a very important and critical role in fetal development because the placenta helps to exchange oxygen, nutrients and waste between the mother and the offspring, therefore, if the mother has any kind of conditions that could affect this process as anemia, preeclampsia, or heart and lung disease this could result in a diminished of pO_2 that may lead the fetus to experience hypoxia.

Fetal hypoxia results in a decrease of amniotic fluid production and a redistribution of fetal blood flow away from peripheral circulation, but an increase of perfusion to the brain, heart and liver (Richardson & Bocking, 1998). Although this redistribution can lead to fetal survival, this experience of adaptation can produce an increased risk of some health condition in adulthood. In animal models it has been reported that maternal hypoxia affects the organogenesis of brain (Northington, Ferriero, Flock, & Martin, 2001) and heart (G. Li et al., 2003; Patterson, Chen, Xue, Xiao, & Zhang, 2010). In the brain, the most vulnerable regions to hypoxic insult are the cerebral cortex, hippocampus and sub-ventricular zone (Northington, Ferriero, Graham,

Traystman, & Martin, 2001). In the hippocampus it was reported that a hypoxic environment induced neuronal death in the CA1 and that cytosolic phospholipase A2 activity plays an important role in the neuronal degeneration mechanism (Arai et al., 2001). Also, inflammation could play a very important role in the mechanism associated with fetal brain injury due to maternal hypoxia. Recently, it was reported that chronic hypoxia exposure induced an increase in the lactate:pyruvate ratio and a decrease in the GSH:GSSG ratio, a favorable pro-oxidant state, and also that the expression levels of Bax, Dcl-2 and p53, which are pro-apoptotic proteins, as well as the levels of some pro-inflammatory cytokines were increased (Guo et al., 2010). In the heart, maternal hypoxia induced atypical expression of important genes that results in modification of the heart structure and function. The expression of PKC ϵ was down-regulated, while the angiotensin II type 2 receptor was up-regulated in the developing heart due to maternal hypoxia, increasing the sensitivity to cardiac ischemic injury in the offspring (Patterson et al., 2010; Xue, Dasgupta, Chen, & Zhang, 2011).

Epigenetics

During fetal development different events occur in a way to repress or activate gene transcription via epigenetic mechanisms. Epigenetic control of gene expression involves modification of the genome without altering the DNA sequence itself. The chromatin-based epigenetic is very important to ensure the correct integration of developmental signals at gene regulatory regions in which chromatin modifications play very important roles. Some of these chromatin modifications are mediated by DNA methylation and histone posttranslational modifications, including histone methylation,

acetylation, phosphorylation, ubiquitylation, sumoylation and propionylation (Jungel, Ospelt, & Gay, 2010; Ouvry-Patat & Schey, 2007). Animal studies have demonstrated the importance of epigenetic changes in mediating the effects on adult phenotype and physiology arising from perturbations of the developmental environment, including maternal diet (Lillicrop, Phillips, Jackson, Hanson, & Burdge, 2005; Waterland & Jirtle, 2003), uterine blood flow (Pham et al., 2003), and maternal nursing behavior (Weaver et al., 2004).

For example, it has been reported that a maternal low-protein diet induced a hypomethylation in the gene promoter region of ACE-1 in the fetal brain (Goyal, Goyal, Leitzke, Gheorghe, & Longo, 2010); likewise the same diet decreased the methylation levels of exon 1₁₀ at glucocorticoids receptor gene promoter and also affect the expression of DNA methyl transferase 1 resultant in an increased expression of GR in the offspring (Lillicrop et al., 2007). Also, it is known that PKC ϵ plays a very important role in cardioprotection during cardiac ischemia and reperfusion injury, however fetal stress in animal models such as hypoxia, nicotine or cocaine exposure resulted in a repression of the PKC ϵ gene in the developing heart causing an increase heart vulnerability to ischemia and reperfusion injuries in the offspring, suggesting a common mechanism of PKC ϵ in fetal programming of heart disease during adulthood (Meyer, Zhang, & Zhang, 2009; Patterson et al., 2010). Moreover, epigenetic regulation has been associated with programming of type 2 diabetes mellitus. In a recent study, where islets were isolated from fetuses exposed previously to IUGR, it was demonstrated that histone acetylation in H3 and H4 at the proximal promoter *Pdx1*, which plays a critical role in the development of the endocrine and exocrine pancreas, was decreased (S. W. Park et al., 2008). This

modification affected the binding of USF1, an activator of *Pdx1*, and the resulting repression of *Pdx1* transcription induced the aberrant development of the pancreas (H. Li et al., 2006).

As we can see, the effects on the offspring of epigenetic changes during development in animals mimic aspects of human disease, for example, metabolic disease, cardiovascular condition, or neurobiological function, and a coherent theory for a role of epigenetic mechanisms in the developmental origins of later chronic disease is emerging.

Hypoxic-ischemic Encephalopathy

One of the most common causes of neonatal brain damage is hypoxic-ischemic encephalopathy due to a systematic asphyxia, which may occur prior, during or after birth (Douglas-Escobar & Weiss, 2012). Epidemiological studies have demonstrated that one to three per 100 live full-term babies will experience HIE, where 15%-20% of them will die in the post natal period and 25% will develop neurological deficits and long-term neurodevelopmental disabilities in later life including, mental retardation, visual motor dysfunction, cerebral palsy and epilepsy (Vannucci & Perlman, 1997). A neonatal animal model for HIE has been developed to mimic this human condition and study the pathophysiology of perinatal HIE which is difficult to perform in a human. This model is known as the Vannucci model in which 7-day postnatal rats experienced a unilateral common carotid ligation followed by systemic hypoxia in 8% oxygen balanced with nitrogen environment (Vannucci et al., 1999). This insult produces permanent hypoxic-ischemic brain damage limited to the cerebral hemisphere ipsilateral to the carotid artery occlusion. Despite the advances in the last two decades in research of cellular processes and molecular mechanism underlying HIE, hypothermia is the only effective treatment

for full-term newborns with mild or moderate HIE, however this treatment has some limitations. The use of the HIE model in this study will help us to evaluate the effect of adverse fetal environment on brain programming disease and also to evaluate possible neuroprotective mechanisms against HIE.

Glucocorticoids

Glucocorticoids are essential for life and play a crucial role in the regulation of growth and development, but also are implicated in various pathogenesis. The glucocorticoids exert their action by binding with different affinity to two receptors, the glucocorticoid and the mineralcorticoid receptor. Interestingly, antagonist effects have been associated with the glucocorticoids, where a neuroprotective and neurodegenerative effect has been reported (Abraham, Harkany, Horvath, & Luiten, 2001). The neurodegenerative effect has been found for long-term exposure and high levels of glucocorticoids while the neuroprotective effect has been observed for physiological levels of glucocorticoids. Also, glucocorticoid receptor has been affected by IUGR, studies with prenatal undernutrition resulting in a decreased expression of hypothalamic glucocorticoid receptors in rats, increasing the risk of hypertension later in life (Perez et al., 2010). In addition, maternal nicotine exposure decreases the mRNA expression levels of hippocampal GR in the offspring (Liu et al., 2012). The neuroprotective role of the glucocorticoid receptors was demonstrated when adult rat brains were pretreated with dexamethasone, a synthetic glucocorticoid, and a reduction of infarct volume after focal cerebral ischemia was observed (Feng, Rhodes, & Bhatt, 2011). A better understanding of molecular mechanisms of neuroprotection by dexamethasone may provide a basis for

the development of newer therapeutic strategies involving corticosteroids or other novel compounds for the prevention and treatment of HI brain injury.

Prostaglandin

Prostaglandin D2 is the most abundant prostaglandin in the brain, a metabolite from arachidonic acid. This PGD2 accumulates in the cerebrospinal fluid, where it induces physiologic sleep in rats and humans. Recently it was reported that PGD2 protects neonatal mouse brain from HI injury (Taniguchi et al., 2007). Also, in-vitro studies using rodent neuronal cells demonstrated that dexamethasone stimulates the gene expression of the 26 kDa glutathione-independent L-PGDS the enzyme responsible for the production of prostaglandin D2 (Garcia-Fernandez et al., 2000). Recent studies demonstrated that PGD2 elicits its downstream effects by activating two G protein-coupled receptors; the DP and chemoattractant receptor-homologous molecule expressed on Th2 cells receptors (hereafter termed DP1 and DP2) where DP1 is coupled to Gas and DP2 is coupled to Gai. Interestingly, these two receptors have divergent effects on cAMP production, when BW245C, a DP1 agonist, is added to hippocampal neurons a rapid increase in cAMP is observed inducing neuroprotection, while when DK-PGD2, a DP2 agonist, is added a decrease in cAMP production was observed resulting in neuronal injury (Liang, Wu, Hand, & Andreasson, 2005). Additionally, it was demonstrated that the activation of ERK1/2 pathway produced neuroprotection in HC2S2 hippocampal neurons subjected to oxygen-glucose deprivation (E. M. Park et al., 2004). Interestingly, ERK1/2 was the major pathway for PGD2-DP receptor-mediated protection in cardiomyocytes (Tokudome et al., 2009).

The Purpose of these Studies

Based upon the theory of programming diseases, which established that adverse maternal environment during the gestational period could affect the development of the fetus and increase the risk to diseases or detrimental health during adulthood, we hypothesized that maternal hypoxia affects the organogenesis of the kidney and the brain and program a hypoxic ischemic encephalopathy sensitive phenotype in neonatal rat brain (figure 1). We began our studies by evaluating how maternal hypoxia affects the development of kidney in fetal and offspring rats and also determine the effect on the expression of angiotensin II type I (AT₁R) and type 2 (AT₂R) receptors (**Chapter 2**). Next, we analyzed the effect of maternal hypoxia on the brain vulnerability to hypoxic-ischemic brain injury in neonatal rats and determine how the expression of glucocorticoid receptors is affected by this maternal insult. The Vannucci model described previously was used to mimic a hypoxic-ischemic brain injury model (**Chapter 3**). Then we studied a possible mechanism associated with the neuroprotective role of dexamethasone against hypoxic-ischemic brain (**Chapter 4**). These studies revealed that 1) maternal hypoxia affects the organogenesis of the kidney and the brain, 2) maternal hypoxia increases the brain vulnerability of neonatal rats against HIE brain injury by repressing the expression of glucocorticoid receptors due to a hypermethylation on the promoter region of GR gene exon1₇ and 1₁₁, and 3) the mechanism of the neuroprotective role of dexamethasone on HIE brain injury is associated with the MAPK/ERK pathway, where the LPGDS/PGD cascade plays an important function.

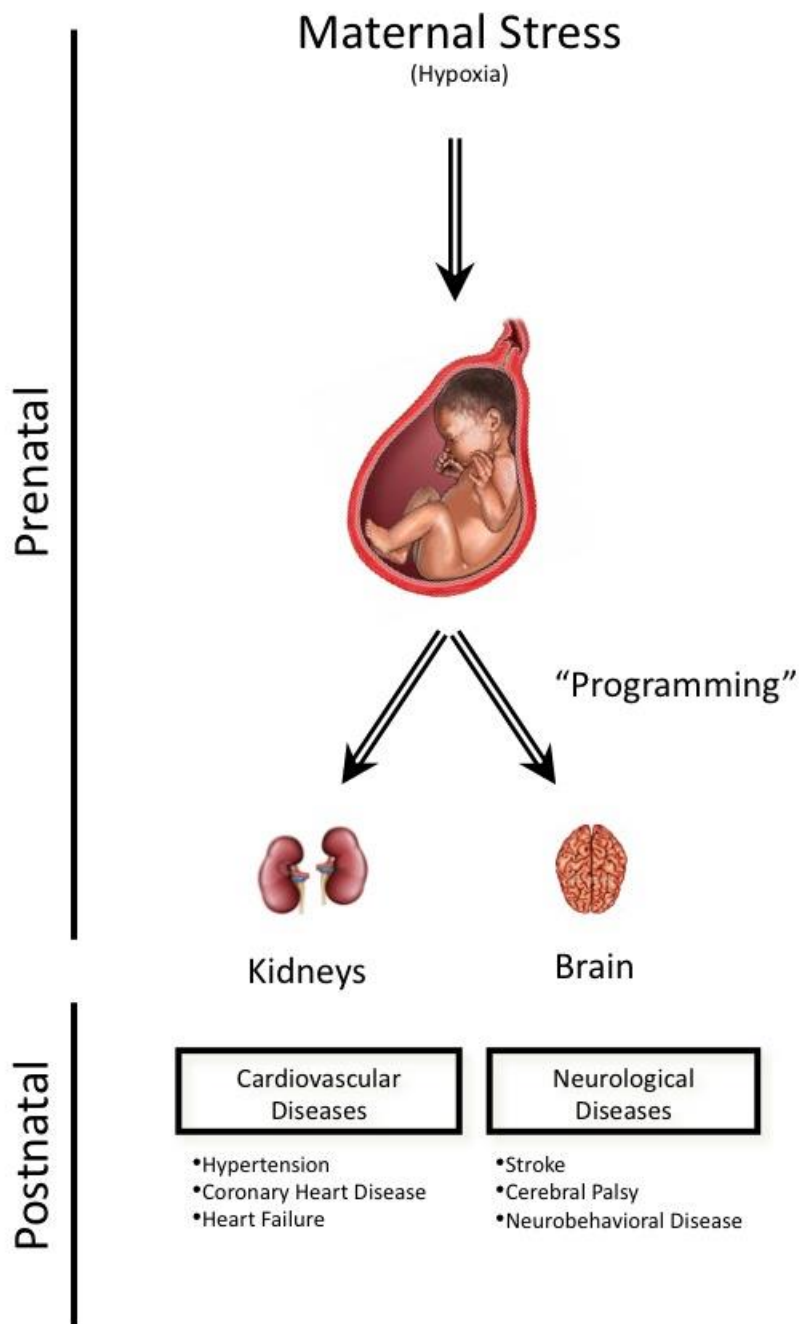


Fig. 1. Developmental programming of health and disease. Maternal stress (hypoxia) impacts normal fetal tissues/organs development and increases the risk of development of cardiovascular, stroke and various neurobehavioral diseases in the offspring.

References

- Abraham, I. M., Harkany, T., Horvath, K. M., & Luiten, P. G. (2001). Action of glucocorticoids on survival of nerve cells: promoting neurodegeneration or neuroprotection? *J Neuroendocrinol*, *13*(9), 749-760.
- Arai, N., Furukawa, N., Miyamae, T., Goshima, Y., Sasaki, Y., Ohshima, E., . . . Misu, Y. (2001). DOPA cyclohexyl ester, a competitive DOPA antagonist, protects glutamate release and resultant delayed neuron death by transient ischemia in hippocampus CA1 of conscious rats. *Neurosci Lett*, *299*(3), 213-216.
- Bruin, J. E., Gerstein, H. C., & Holloway, A. C. (2010). Long-term consequences of fetal and neonatal nicotine exposure: a critical review. *Toxicol Sci*, *116*(2), 364-374. doi: 10.1093/toxsci/kfq103
- Douglas-Escobar, M., & Weiss, M. D. (2012). Biomarkers of hypoxic-ischemic encephalopathy in newborns. *Front Neurol*, *3*, 144. doi: 10.3389/fneur.2012.00144
- Dudley, K. J., Li, X., Kobor, M. S., Kippin, T. E., & Bredy, T. W. (2011). Epigenetic mechanisms mediating vulnerability and resilience to psychiatric disorders. *Neurosci Biobehav Rev*, *35*(7), 1544-1551. doi: 10.1016/j.neubiorev.2010.12.016
- Elahi, M. M., Cagampang, F. R., Mukhtar, D., Anthony, F. W., Ohri, S. K., & Hanson, M. A. (2009). Long-term maternal high-fat feeding from weaning through pregnancy and lactation predisposes offspring to hypertension, raised plasma lipids and fatty liver in mice. *The British journal of nutrition*, *102*(4), 514-519. doi: 10.1017/S000711450820749X
- Erkkola, M., Nwaru, B. I., & Viljakainen, H. T. (2011). Maternal vitamin D during pregnancy and its relation to immune-mediated diseases in the offspring. *Vitamins and hormones*, *86*, 239-260. doi: 10.1016/B978-0-12-386960-9.00010-1
- Feng, Y., Rhodes, P. G., & Bhatt, A. J. (2011). Dexamethasone pre-treatment protects brain against hypoxic-ischemic injury partially through up-regulation of vascular endothelial growth factor A in neonatal rats. *Neuroscience*, *179*, 223-232. doi: 10.1016/j.neuroscience.2011.01.050
- Garcia-Fernandez, L. F., Iniguez, M. A., Eguchi, N., Fresno, M., Urade, Y., & Munoz, A. (2000). Dexamethasone induces lipocalin-type prostaglandin D synthase gene expression in mouse neuronal cells. *J Neurochem*, *75*(2), 460-470.
- Gniuli, D., Calcagno, A., Caristo, M. E., Mancuso, A., Macchi, V., Mingrone, G., & Vettor, R. (2008). Effects of high-fat diet exposure during fetal life on type 2

- diabetes development in the progeny. *Journal of lipid research*, 49(9), 1936-1945. doi: 10.1194/jlr.M800033-JLR200
- Golan, H., & Huleihel, M. (2006). The effect of prenatal hypoxia on brain development: short- and long-term consequences demonstrated in rodent models. *Dev Sci*, 9(4), 338-349. doi: 10.1111/j.1467-7687.2006.00498.x
- Goyal, R., Goyal, D., Leitzke, A., Gheorghe, C. P., & Longo, L. D. (2010). Brain renin-angiotensin system: fetal epigenetic programming by maternal protein restriction during pregnancy. *Reprod Sci*, 17(3), 227-238. doi: 10.1177/1933719109351935
- Gressens, P., Mesples, B., Sahir, N., Marret, S., & Sola, A. (2001). Environmental factors and disturbances of brain development. *Semin Neonatol*, 6(2), 185-194. doi: 10.1053/siny.2001.0048
- Guo, R., Hou, W., Dong, Y., Yu, Z., Stites, J., & Weiner, C. P. (2010). Brain injury caused by chronic fetal hypoxemia is mediated by inflammatory cascade activation. *Reprod Sci*, 17(6), 540-548. doi: 10.1177/1933719110364061
- Harris, A., & Seckl, J. (2011). Glucocorticoids, prenatal stress and the programming of disease. *Horm Behav*, 59(3), 279-289. doi: 10.1016/j.yhbeh.2010.06.007
- Hughson, M. D., Douglas-Denton, R., Bertram, J. F., & Hoy, W. E. (2006). Hypertension, glomerular number, and birth weight in African Americans and white subjects in the southeastern United States. *Kidney Int*, 69(4), 671-678. doi: 10.1038/sj.ki.5000041
- Jungel, A., Ospelt, C., & Gay, S. (2010). What can we learn from epigenetics in the year 2009? *Curr Opin Rheumatol*, 22(3), 284-292. doi: 10.1097/BOR.0b013e3283389641
- Koleganova, N., Piecha, G., Ritz, E., Becker, L. E., Muller, A., Weckbach, M., . . . Gross-Weissmann, M. L. (2011). Both high and low maternal salt intake in pregnancy alter kidney development in the offspring. *Am J Physiol Renal Physiol*, 301(2), F344-354. doi: 10.1152/ajprenal.00626.2010
- Li, G., Xiao, Y., Estrella, J. L., Ducsay, C. A., Gilbert, R. D., & Zhang, L. (2003). Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *Journal of the Society for Gynecologic Investigation*, 10(5), 265-274.
- Li, H., Rauch, T., Chen, Z. X., Szabo, P. E., Riggs, A. D., & Pfeifer, G. P. (2006). The histone methyltransferase SETDB1 and the DNA methyltransferase DNMT3A interact directly and localize to promoters silenced in cancer cells. *J Biol Chem*, 281(28), 19489-19500. doi: 10.1074/jbc.M513249200

- Li, Y., Xiao, D., Dasgupta, C., Xiong, F., Tong, W., Yang, S., & Zhang, L. (2012). Perinatal nicotine exposure increases vulnerability of hypoxic-ischemic brain injury in neonatal rats: role of angiotensin II receptors. *Stroke*, *43*(9), 2483-2490. doi: 10.1161/STROKEAHA.112.664698
- Liang, X., Wu, L., Hand, T., & Andreasson, K. (2005). Prostaglandin D2 mediates neuronal protection via the DP1 receptor. *J Neurochem*, *92*(3), 477-486. doi: 10.1111/j.1471-4159.2004.02870.x
- Lillicrop, K. A., Phillips, E. S., Jackson, A. A., Hanson, M. A., & Burdge, G. C. (2005). Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr*, *135*(6), 1382-1386.
- Lillicrop, K. A., Slater-Jefferies, J. L., Hanson, M. A., Godfrey, K. M., Jackson, A. A., & Burdge, G. C. (2007). Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications. *The British journal of nutrition*, *97*(6), 1064-1073. doi: 10.1017/S000711450769196X
- Liu, L., Liu, F., Kou, H., Zhang, B. J., Xu, D., Chen, B., . . . Wang, H. (2012). Prenatal nicotine exposure induced a hypothalamic-pituitary-adrenal axis-associated neuroendocrine metabolic programmed alteration in intrauterine growth retardation offspring rats. *Toxicol Lett*, *214*(3), 307-313. doi: 10.1016/j.toxlet.2012.09.001
- Mackay, H., Khazall, R., Patterson, Z. R., Wellman, M., & Abizaid, A. (2013). Rats perinatally exposed to food restriction and high-fat diet show differences in adipose tissue gene expression under chronic caloric restriction. *Adipocyte*, *2*(4), 237-245. doi: 10.4161/adip.24752
- Mackenzie, H. S., & Brenner, B. M. (1995). Fewer nephrons at birth: a missing link in the etiology of essential hypertension? *Am J Kidney Dis*, *26*(1), 91-98.
- Maier, S. E., Chen, W. J., Miller, J. A., & West, J. R. (1997). Fetal alcohol exposure and temporal vulnerability regional differences in alcohol-induced microencephaly as a function of the timing of binge-like alcohol exposure during rat brain development. *Alcohol Clin Exp Res*, *21*(8), 1418-1428.
- Manalich, R., Reyes, L., Herrera, M., Melendi, C., & Fundora, I. (2000). Relationship between weight at birth and the number and size of renal glomeruli in humans: a histomorphometric study. *Kidney Int*, *58*(2), 770-773. doi: 10.1046/j.1523-1755.2000.00225.x

- McDade, T. W., Beck, M. A., Kuzawa, C. W., & Adair, L. S. (2001). Prenatal undernutrition and postnatal growth are associated with adolescent thymic function. *J Nutr*, *131*(4), 1225-1231.
- McEwen, B. S. (2008). Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators. *Eur J Pharmacol*, *583*(2-3), 174-185. doi: 10.1016/j.ejphar.2007.11.071
- Meyer, K., Zhang, H., & Zhang, L. (2009). Direct effect of cocaine on epigenetic regulation of PKCepsilon gene repression in the fetal rat heart. *J Mol Cell Cardiol*, *47*(4), 504-511. doi: 10.1016/j.yjmcc.2009.06.004
- Moritz, K. M., Cuffe, J. S., Wilson, L. B., Dickinson, H., Wlodek, M. E., Simmons, D. G., & Denton, K. M. (2010). Review: Sex specific programming: a critical role for the renal renin-angiotensin system. *Placenta*, *31 Suppl*, S40-46. doi: 10.1016/j.placenta.2010.01.006
- Nelson, E. A., & Taylor, B. J. (2001). International Child Care Practices Study: infant sleep position and parental smoking. *Early Hum Dev*, *64*(1), 7-20.
- Northington, F. J., Ferriero, D. M., Flock, D. L., & Martin, L. J. (2001). Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *21*(6), 1931-1938.
- Northington, F. J., Ferriero, D. M., Graham, E. M., Traystman, R. J., & Martin, L. J. (2001). Early Neurodegeneration after Hypoxia-Ischemia in Neonatal Rat Is Necrosis while Delayed Neuronal Death Is Apoptosis. *Neurobiol Dis*, *8*(2), 207-219. doi: 10.1006/nbdi.2000.0371
- O'Sullivan, L., Cuffe, J. S., Paravicini, T. M., Campbell, S., Dickinson, H., Singh, R. R., . . . Moritz, K. M. (2013). Prenatal exposure to dexamethasone in the mouse alters cardiac growth patterns and increases pulse pressure in aged male offspring. *PLoS One*, *8*(7), e69149. doi: 10.1371/journal.pone.0069149
- Ortiz, L. A., Quan, A., Zarzar, F., Weinberg, A., & Baum, M. (2003). Prenatal dexamethasone programs hypertension and renal injury in the rat. *Hypertension*, *41*(2), 328-334.
- Oster, M., Murani, E., Metges, C. C., Ponsuksili, S., & Wimmers, K. (2012). A gestational high protein diet affects the abundance of muscle transcripts related to cell cycle regulation throughout development in porcine progeny. *PLoS One*, *7*(4), e34519. doi: 10.1371/journal.pone.0034519

- Ouvry-Patat, S. A., & Schey, K. L. (2007). Characterization of antimicrobial histone sequences and posttranslational modifications by mass spectrometry. *J Mass Spectrom*, *42*(5), 664-674. doi: 10.1002/jms.1200
- Park, E. M., Joh, T. H., Volpe, B. T., Chu, C. K., Song, G., & Cho, S. (2004). A neuroprotective role of extracellular signal-regulated kinase in N-acetyl-O-methyl-dopamine-treated hippocampal neurons after exposure to in vitro and in vivo ischemia. *Neuroscience*, *123*(1), 147-154.
- Park, S. W., Davison, J. M., Rhee, J., Hruban, R. H., Maitra, A., & Leach, S. D. (2008). Oncogenic KRAS induces progenitor cell expansion and malignant transformation in zebrafish exocrine pancreas. *Gastroenterology*, *134*(7), 2080-2090. doi: 10.1053/j.gastro.2008.02.084
- Patterson, A. J., Chen, M., Xue, Q., Xiao, D., & Zhang, L. (2010). Chronic prenatal hypoxia induces epigenetic programming of PKC{epsilon} gene repression in rat hearts. *Circulation research*, *107*(3), 365-373. doi: 10.1161/CIRCRESAHA.110.221259
- Perez, H., Soto-Moyano, R., Ruiz, S., Hernandez, A., Sierralta, W., Olivares, R., . . . Flores, F. J. (2010). A putative role for hypothalamic glucocorticoid receptors in hypertension induced by prenatal undernutrition in the rat. *Neurosci Lett*, *483*(1), 41-46. doi: 10.1016/j.neulet.2010.07.057
- Pham, T. D., MacLennan, N. K., Chiu, C. T., Laksana, G. S., Hsu, J. L., & Lane, R. H. (2003). Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. *Am J Physiol Regul Integr Comp Physiol*, *285*(5), R962-970. doi: 10.1152/ajpregu.00201.2003
- Richardson, B. S., & Bocking, A. D. (1998). Metabolic and circulatory adaptations to chronic hypoxia in the fetus. *Comp Biochem Physiol A Mol Integr Physiol*, *119*(3), 717-723.
- Singh, R. R., Cullen-McEwen, L. A., Kett, M. M., Boon, W. M., Dowling, J., Bertram, J. F., & Moritz, K. M. (2007). Prenatal corticosterone exposure results in altered AT1/AT2, nephron deficit and hypertension in the rat offspring. *J Physiol*, *579*(Pt 2), 503-513. doi: 10.1113/jphysiol.2006.125773
- Stein, C. E., Kumaran, K., Fall, C. H., Shaheen, S. O., Osmond, C., & Barker, D. J. (1997). Relation of fetal growth to adult lung function in south India. *Thorax*, *52*(10), 895-899.
- Strang-Karlsson, S., Raikkonen, K., Pesonen, A. K., Kajantie, E., Paavonen, E. J., Lahti, J., . . . Andersson, S. (2008). Very low birth weight and behavioral symptoms of attention deficit hyperactivity disorder in young adulthood: the Helsinki study of

very-low-birth-weight adults. *Am J Psychiatry*, 165(10), 1345-1353. doi: 10.1176/appi.ajp.2008.08010085

Taniguchi, H., Mohri, I., Okabe-Arahoru, H., Aritake, K., Wada, K., Kanekiyo, T., . . . Taniike, M. (2007). Prostaglandin D2 protects neonatal mouse brain from hypoxic ischemic injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(16), 4303-4312. doi: 10.1523/JNEUROSCI.0321-07.2007

Tokudome, S., Sano, M., Shinmura, K., Matsubashi, T., Morizane, S., Moriyama, H., . . . Fukuda, K. (2009). Glucocorticoid protects rodent hearts from ischemia/reperfusion injury by activating lipocalin-type prostaglandin D synthase-derived PGD2 biosynthesis. *J Clin Invest*, 119(6), 1477-1488. doi: 10.1172/JCI37413

Vannucci, R. C., Connor, J. R., Mauger, D. T., Palmer, C., Smith, M. B., Towfighi, J., & Vannucci, S. J. (1999). Rat model of perinatal hypoxic-ischemic brain damage. *J Neurosci Res*, 55(2), 158-163.

Vannucci, R. C., & Perlman, J. M. (1997). Interventions for perinatal hypoxic-ischemic encephalopathy. *Pediatrics*, 100(6), 1004-1014.

Waterland, R. A., & Jirtle, R. L. (2003). Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol*, 23(15), 5293-5300.

Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., . . . Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nat Neurosci*, 7(8), 847-854. doi: 10.1038/nm1276

Wlodek, M. E., Westcott, K., Siebel, A. L., Owens, J. A., & Moritz, K. M. (2008). Growth restriction before or after birth reduces nephron number and increases blood pressure in male rats. *Kidney Int*, 74(2), 187-195. doi: 10.1038/ki.2008.153

Woods, L. L., Ingelfinger, J. R., Nyengaard, J. R., & Rasch, R. (2001). Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatr Res*, 49(4), 460-467. doi: 10.1203/00006450-200104000-00005

Xiao, D., Huang, X., Xu, Z., Yang, S., & Zhang, L. (2009). Prenatal cocaine exposure differentially causes vascular dysfunction in adult offspring. *Hypertension*, 53(6), 937-943. doi: 10.1161/HYPERTENSIONAHA.108.121830

Xiao, D., Huang, X., Yang, S., & Zhang, L. (2011). Antenatal nicotine induces heightened oxidative stress and vascular dysfunction in rat offspring. *Br J Pharmacol*, 164(5), 1400-1409. doi: 10.1111/j.1476-5381.2011.01437.x

Xue, Q., Dasgupta, C., Chen, M., & Zhang, L. (2011). Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. *Cardiovasc Res*, 89(2), 300-308. doi: 10.1093/cvr/cvq303

CHAPTER TWO
FETAL HYPOXIA RESULTS IN PROGRAMMING OF
ABERRANT ANGIOTENSIN II RECEPTOR EXPRESSION
PATTERNS AND KIDNEY DEVELOPMENT

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Abstract

The present study tested the hypothesis that fetal hypoxia adversely affects kidney development in fetal and offspring rats and alter the expression patterns of angiotensin II type 1 and type 2 receptors. Time-dated pregnant rats were divided between normoxic and hypoxic (10.5% O₂ last period of gestation) groups. Protein expression, in the pups, was determined using western blot. Hypoxic treatment significantly decreased body and kidney weight in 21-day fetuses and 7-day neonates. In 3-month-old offspring there were no significant differences in body and kidney weight between hypoxic and control animals. Fetal hypoxia had no effect on kidney AT₁R density in E21 or P7, but significantly decreased kidney AT₁R protein abundance in both male and female adults. In contrast, kidney AT₂R density was not affected by fetal hypoxia throughout the developmental stages studied. The hypoxia-mediated reduction of nephron numbers was progressively from P7 worsened into the adulthood with females affected more than males. The results suggest that fetal hypoxia causes programming of aberrant kidney development and accelerates the aging process of the kidney during the postnatal development, which may contribute to an increased risk of cardiovascular disease.

Introduction

The most critical period of the human being is during fetal development, the stage in which the mother could expose the fetus to adverse environment that could have a long-term effect during adulthood. The studies of Barker and colleagues are the fundament for what is known as developmental programming of diseases in which they found an inverse relationship between birth weight and cardiovascular diseases later in the adulthood (Barker, 1995, 1997). Two of the most common challenges to the fetus during development are reduction in oxygen and nutrient delivery. There are various maternal stressors that could affect body weight in the offspring, for example maternal protein-restriction diet (Woods, Ingelfinger, Nyengaard, & Rasch, 2001), nicotine exposure (Lawrence et al., 2008), intrauterine malnutrition (Zhang et al., 2000), and hypoxia (Julian et al., 2007).

Retardation of renal development that occurs in individuals of low birth weight increases the risk of renal diseases during adulthood (Brenner & Chertow, 1994). The process of nephrogenesis in the rats starts during mid-gestation and finishes after post-natal day 10 (Larsson, Aperia, & Wilton, 1980). Previous studies demonstrated that maternal low-protein diet in rats resulted in low birth weight, suppressed the renal renin-angiotensin system, and reduced the number of nephrons, leading to hypertension in offspring (Woods & Rasch, 1998). The aging process of the kidney is associated with physiological and functional changes, including reduced kidney weight and glomerular number, and changes in the shape of glomeruli and the formation of glomerular arterioles (Weinstein & Anderson, 2010). These changes in combination with decrease in renal function lead to hypertension and other cardiovascular diseases. The RAS is associated with one of the mechanisms that are involved in developmental problems in fetal organs,

including the kidney (Tufro-McReddie, Romano, Harris, Ferder, & Gomez, 1995). Angiotensin II is the primary peptide in the system, which activates two subtypes of G-protein coupled receptors: angiotensin II type 1 and type 2 receptors. The AT₁R plays a key role in the regulation of blood pressure, hormone secretion and renal function while AT₂R is involved mainly in the development and growth (de Gasparo, Catt, Inagami, Wright, & Unger, 2000).

Little is known about the effect of common fetal stress of hypoxia on the kidney development. Therefore, in the present study, we determined the effect of fetal hypoxia on the glomerular development and the expression patterns of AT₁R and AT₂R in the kidney in three developmental stages of near-term fetal, neonatal, and adult rats. The results suggest that fetal hypoxia causes programming of aberrant kidney development and accelerates the aging process of the kidney during the postnatal development, which may contribute to an increased risk of cardiovascular disease, particularly hypertension in offspring.

Material and Methods

Experimental Animals and Hypoxic Exposure

Time-dated pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI) and were randomly divided into normoxic control group and continuous hypoxic exposure group. The hypoxic group was exposed to 10.5% oxygen from day 15 to day 21 of gestation, a period comparable with the third trimester of gestation in humans. Hypoxia was induced to the pregnant rats by a mixture of nitrogen gas and air in individual chambers as described previously (G. Li et al., 2003). The normoxic control group was housed identically with room air flowing through chambers.

Water and food were provided as desired. Some of the pregnant rats, of both groups, were allowed to give birth naturally, and no fetal loss in the normoxia or hypoxia groups was observed. The offspring were allowed to lactation and no further treatment before euthanasia at postnatal day 7 and 3 month-old. The other pregnant dams were sacrificed on day 21 of gestation and fetuses were collected for studies. Kidneys were isolated and stored at -80°C . Body and kidney weight was determined. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Immunoblotting

Protein abundance of renal AT_1R and AT_2R was determined with Western blot analysis as reported previously (Mao et al., 2010). Briefly, kidneys were homogenized in an ice-cold lysis buffer (20 mM HEPES, 10 mM KCl, 1.5 mM MgCl_2 , 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 2 $\mu\text{g}/\text{ml}$ aprotinin, pH 7.4). Homogenates were centrifuged at 4°C for 10 min at 14,000g, and supernatants aliquots were collected and stored at -80°C . Protein concentrations were determined using a protein assay kit from Bio-Rad. Samples with equal protein (40 μg) were loaded on 7.5% sodium dodecyl sulfate polyacrylamide gel and performed the electrophoresis analysis. After electrophoresis, proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked in TBST containing 5% dry milk for 60 min at room temperature. The membranes were incubated with rabbit AT_1R and AT_2R polyclonal antibody (1:1000 and 1:2000 dilution respectively; Santa Cruz Biotechnology,

Santa Cruz, CA) overnight at 4°C. The membranes were then washed and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:4000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized with enhanced chemiluminescence reagents, and the blots were exposed to Hyperfilm (GE Healthcare). Results were analyzed and quantified by the Kodak electrophoresis documentation and analysis system with Kodak ID image analysis software. For comparison of the levels of AT₁R and AT₂R protein relative density between the groups, samples were normalized first to GAPDH values and then presented as fold values relative to sham-treated animals.

RT-PCR

Three month-old rat kidney RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and subjected to reverse transcription with Superscript III First-Strand Synthesis System (Invitrogen), following the manufacturer's instructions. Briefly, 5 µg total RNA was reverse transcribed into cDNA in a 20 µl volume reaction following the protocol of 50°C for 50 minutes and 85°C for 5 minutes. The mRNA abundance of AT1aR and AT1bR was measured with real-time PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) as described previously and amplification of GAPDH was used as internal reference. Primers used for AT1aR mRNA: 5'-ccattcaccctgcctcag-3' (forward) and 5'-acggcttgcttggttactc-3' (reverse) and for AT1bR mRNA: 5'-atgtctccagtcacctctca-3' (forward) and 5'-tgacctcccatctcctttg-3' (reverse). Real-time PCR was performed in a final volume of 25 µl and each PCR reaction mixture consisted of 500 nM of primers and iQ SYBR Green Supermix containing 0.625 unit hot-start Taq polymerase, 400 µM each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM

ammonium sulfate, 40 mM Tris-HCl, 6 mM MgSO₄, SYBR Green I, 20 nM fluorescing and stabilizers. We used the following real time-PCR protocol: 95°C for 10 minutes, followed by 40 cycles of 95°C for 10 seconds, annealing for 10 seconds at appropriate temperature depending on the primer sequence, 72°C for 10 seconds. Serial dilutions of the positive control were done on each plate to create a standard curve for the quantification.

Glomerular Counting

The number of glomeruli per kidney was determined for P7 and 3M female and male rats as previously described (Boubred et al., 2007). Briefly, kidneys were removed and weighed. Whole kidneys were incubated in 50% hydrochloric acid for 45 min at 37°C, the incubation time being dependent on kidney weight. Kidneys were rinsed with tap water and stored overnight at 4°C in a gauged flask. Following mechanical dissociation, tubules and glomeruli were suspended in 10 ml of distilled water. Three 0.5-ml aliquots were obtained from each kidney homogenate and placed in a hemocytometer-like chamber, and glomeruli were counted under microscope by two investigators who were unaware of the specimen origin. The three results were averaged, and then the value was used to determine the total number of glomeruli in the sample and therefore in the kidney.

Statistical Analysis

Results are expressed as mean \pm standard error of mean. Experimental number (n) represents fetuses and offspring from different dams. Statistical comparisons for the body and kidney weight, the kidney to body weight ratio, and the number of glomeruli

were conducted by two-way ANOVA followed by a Bonferroni test. Differences in AT₁R or AT₂R protein abundance between normoxic and hypoxic groups were compared using unpaired t-test analysis. In all cases the differences were considered significant when $P < 0.05$.

Results

Effect of Fetal Hypoxia on Body and Kidney Weight

Fetal hypoxia resulted in a significant decrease in body and kidney weight in E21 and P7 rats (Figure 1A, 1B). The kidney-to-body weight ratio was significantly higher in P7 rats than that in E21 rats (Figure 1C), indicating a continuous growth and maturation of the kidney in neonatal rats. Hypoxia treatment decreased the kidney-to-body weight ratio in E21 rats but increased it in P7 rats (Figure 1C). In 3M offspring, there were no significant differences in body and kidney weight in either males or females between the control and hypoxic-treated animals (Figures 1D and 1E). The kidney-to-body weight ratio was not significantly different between males and females in adult offspring, but both were significantly decreased as compared with that in P7 neonates (Figures 1C and 1F). Fetal hypoxia had no significant effect on the kidney-to-body weight ratio in either male or female adult offspring (Figure 1F).

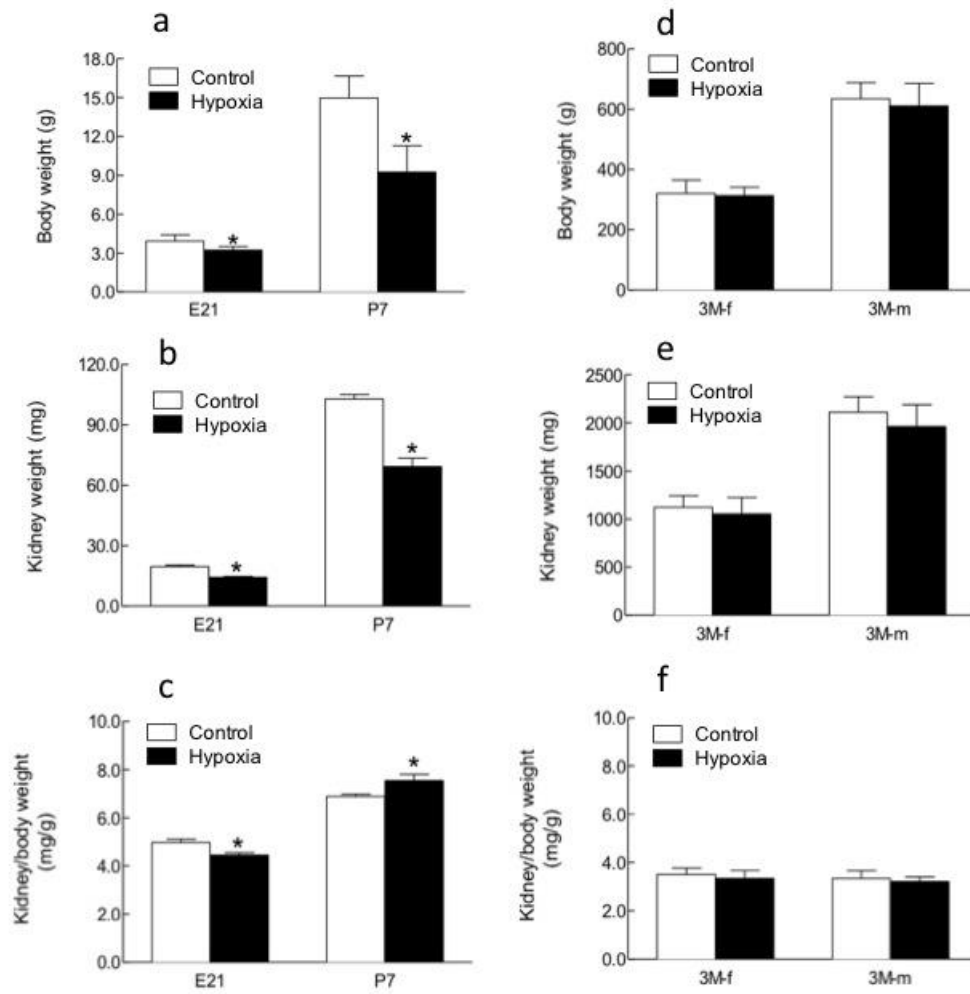


Figure 1. Effect of maternal hypoxia exposure on body weight (a), kidney weight (b) and in the kidney/body weight ratio (c) in fetus of embryonic day 21 (E21) and post-natal day 7 (P7) offspring and on body weight (d), kidney weight (e) and in the kidney/body weight ratio (f) in 3 month-old females (3M-f) and 3 month-old males (3M-m) offspring. *P < 0.05; N=12.

Effect of Fetal Hypoxia on Renal AT₁R and AT₂R Protein and mRNA Abundance

The protein abundance of AT₁R and AT₂R in the kidney was determined by Western blot analysis. As shown in figure 2, AT₁R protein abundance in the kidney was not significantly different in either fetal or neonatal rats between the control and hypoxic-treated animals. However, in adult offspring, AT₁R protein abundance in the kidney was significantly decreased in both males (43%) and females (62%) that had exposed to hypoxia before birth, as compared with the control animals (Figure 2). In contrast, fetal hypoxia had no significant effect on AT₂R protein abundance in the kidney at all three developmental stages examined (Figure 4) Consistent with the finding of protein abundance for 3M offspring, fetal hypoxia exposure caused a significant decrease in AT₁R mRNA abundance in the kidney of both male and female (Figure 3). Similar results were obtained on the ratio between kidney AT₁R and AT₂R where the fetal hypoxia decrease this ratio for male and female adult offspring (Figure 5).

Effects of Fetal Hypoxia on Glomerular Number

Figure 4 shows the effect of fetal hypoxia on total number of glomeruli per kidney in neonates and adult offspring. In P7 neonates, there was a small but significant decrease in kidney glomerular numbers in hypoxic-treated animals, as compared with the normoxic pups (Figure 6). This hypoxia-mediated reduction of nephron numbers was progressively worsened into the adulthood with females (52% reduction) affected more than males (26% reduction) (Figure 6).

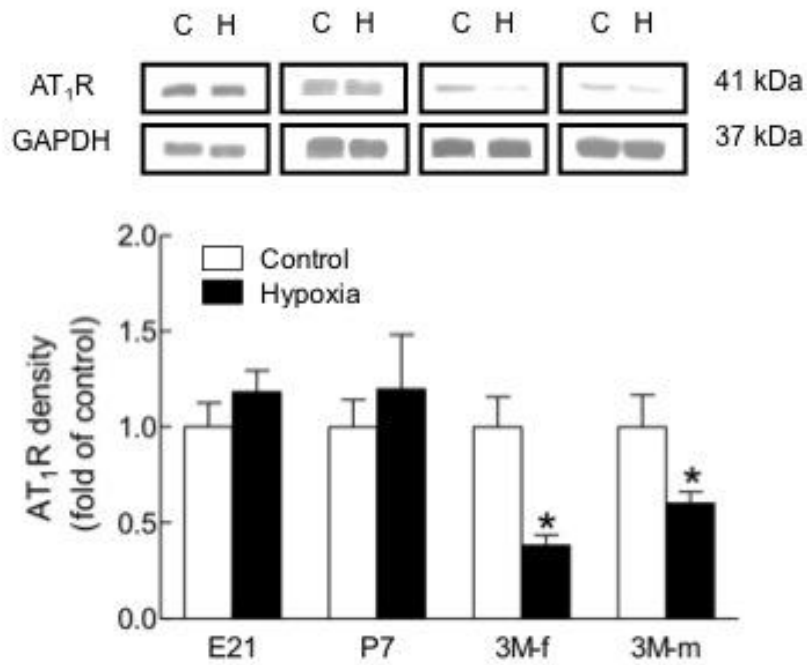


Figure 2. AT₁R protein expression on kidney of fetus (E21), post-natal day 7(P7), male and female 3 month-old (3M-f and 3M-m) after maternal hypoxia and normoxia. Protein levels were determined by Western blots (C – control; H – hypoxia). *P < 0.05; N=5.

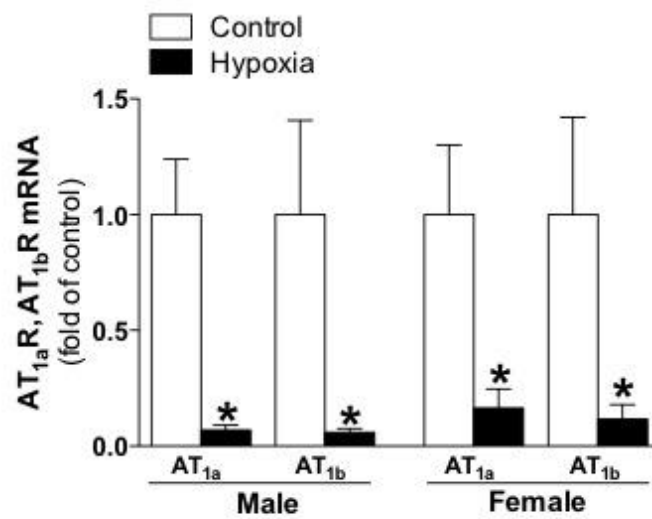


Figure 3. Relative expression of AT_{1a} and AT_{1b} receptor mRNA. Male and female adult offspring rats that were exposed to fetal hypoxia during the last week of gestational period. Real-time PCR was performed using tissue from the left kidneys of these animals. *P < 0.05; N=5.

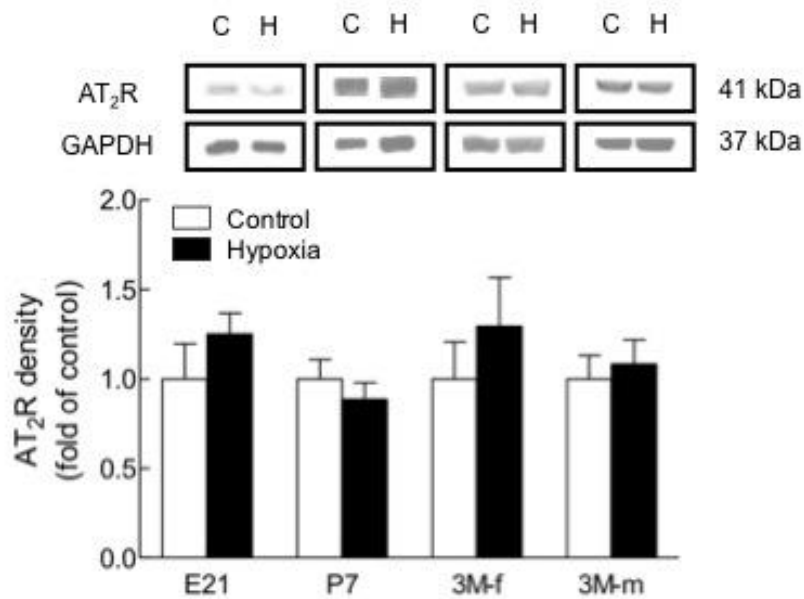


Figure 4. Effect of maternal hypoxia exposure on protein expression of AT₂R in the kidney for fetus (E21), post-natal day 7 (P7), male and female 3 month-old (3M-f and 3M-m) offspring. Protein levels were determined by Western blots (C – control; H – hypoxia). *P < 0.05; N=5.

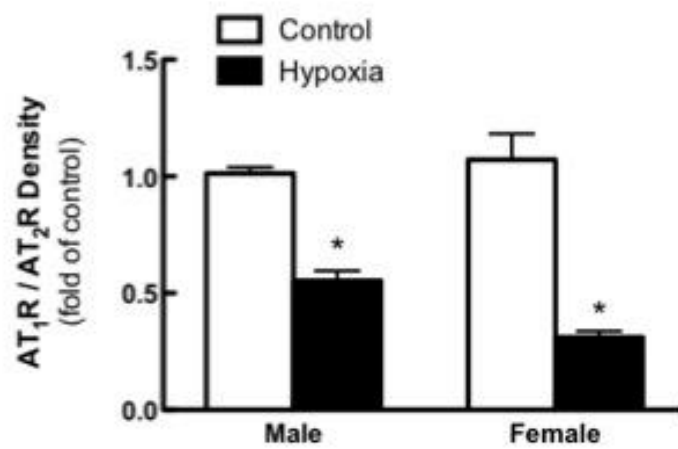


Figure 5. Effect of maternal hypoxia exposure on kidney AT₁R/AT₂R protein expression ratio in male and female adult offspring. *P < 0.05; N=5.

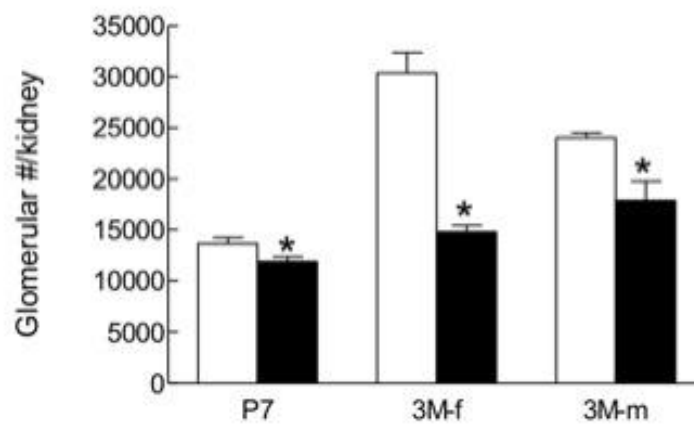


Figure 6. Glomerular number in offspring post-natal day 7 (P7), female and male 3 month-old (3M-f and 3M-m) of mothers maintained on normoxia throughout pregnancy and hypoxia during last week of gestation. Control represents the normoxia group (□); hypoxia represents the group exposed to treatment (■). *P < 0.05 N=5.

Discussion

There is plenty of evidence that correlates low birth weight in term gestation with an increased risk of cardiovascular disease during adulthood (Eriksson et al., 1999). The present finding that fetal hypoxia caused a significant decrease in the body weight of fetal and neonatal rats is consistent with the previous studies (Tapanainen et al., 1994). No significant difference was observed in the adult offspring between normoxia and hypoxia-treated animals in the present study, suggesting “catch-up” growth during postnatal development in hypoxia-treated animals. A number of studies have shown that *in utero* undernutrition causes fetal growth restriction and low birth weight, which is associated with “catch-up” growth during postnatal development (Chen, Martin-Gronert, Tarry-Adkins, & Ozanne, 2009; Woods, Weeks, & Rasch, 2004). These studies suggest a common response of decreased body weight in early developmental stage and “catch-up” growth in postnatal development in fetal malnutrition and fetal hypoxia models. It has been shown that low birth weight associated with accelerated postnatal growth is a trigger for development of adult disease and ultimately can affect longevity (Woods, Ingelfinger, & Rasch, 2005).

In the present study, fetal hypoxia resulted in a decrease in the kidney weight in the fetus and neonate. However in adult offspring, the kidney weight was not significantly different between the control and hypoxic-treated animals in both females and males. Interesting, perinatal nicotine exposure affected the kidney weight not just in the fetus and neonate but also in female and male adult offspring (Mao et al., 2009). The finding that the kidney-to-body weight ratio was significantly greater in P7 rats than that in E21 rats indicates a continuous growth and maturation of the kidney in neonatal rats. This is consistent with the previous finding that the process of nephrogenesis in the rats

starts during mid-gestation and finishes after post-natal day 10 (Larsson et al., 1980). The finding that the kidney-to-body weight ratio was significantly decreased in adult rats as compared with that in neonatal rats is intriguing and suggests asymmetric growth and aging process of reduced kidney weight during the postnatal development¹¹. Hypoxia decreased the kidney-to-body weight ratio in E21 rats. It has been demonstrated that fetal hypoxia results in a decrease in amniotic fluid production and redistribution of fetal blood flow with a decrease in perfusion to the kidney and an increase of perfusion to the brain, heart and liver (Luzi et al., 1996). The finding of the increased kidney-to-body weight ratio in P7 rats in hypoxic animals suggests a compensatory growth of the kidney in neonates. Similar to the present finding, maternal protein restriction caused a decrease in the kidney-to-body weight ratio in the newborn and P1 offspring, but in P5 there was no difference in the ratio between the low protein diet and control groups (Woods et al., 2004).

Abnormal development of the kidney may affect adult cardiovascular and renal function. Brenner *et al.* (Brenner, Garcia, & Anderson, 1988) introduced the hypothesis that due to a lower nephron number the surface area of filtration is smaller, leading to an increase in glomerular pressure and systematic hypertension. We demonstrated in the present study that fetal hypoxia decreased the nephron number in the offspring as early as P7 neonatal rats. In figure 6 the difference observed in the number of glomeruli between normoxia and hypoxia groups for P7 is less than that observed in adults. It is generally perceived, at least in human, that the number of glomeruli does not change after birth but as mentioned above the nephrogenesis process for rats started during mid-gestation and finishes after P10 whereby the maturation process still has not completed and therefore

the number of nephrons for normoxia group (P7) is smaller than the observed on adults. Although the nephron number would not change during adulthood however the total glomerular volume may increase during adulthood due to fully-perfused glomeruli. As the kidney matures, more glomeruli will become functional as reflected by an increase in GFR. In figure 1, there is no difference in the kidney-to-body weight ratio for adults rat, even though the number of glomeruli is lower for the hypoxia group as demonstrated in figure 6. This observation could be due to glomerular hypertrophy that maintain the renal filtration surface area, which may in turn lead to adverse effects in the kidney as a result of chronic glomerular hyperfiltration (Hostetter, 2003).

There is plenty of evidence regarding the existence of a relationship between lower nephron number and hypertension in humans (Keller, Zimmer, Mall, Ritz, & Amann, 2003) and in rats (Gray, Denton, Cullen-McEwen, Bertram, & Moritz, 2010). Several different animal models including maternal alcohol administration and high salt diet during pregnancy demonstrated the reduced nephron number and increased blood pressure in adult offspring (Gray et al., 2010). Woods *et al.* reported that the maternal protein restriction decreased the number of nephrons in adult kidneys. Interesting, they found that although the degree of hypertension in male and female adult rats was similar, the number of nephrons in males was lower than that in females (Woods et al., 2004). In contrast to the findings in animal models of low protein diet and high salt diet (Woods et al., 2004), fetal hypoxia resulted in a greater reduction in the nephron number in female (52%) than male (26%) adult offspring. These findings suggest differential effects of fetal insults on gender-specific programming of aberrant nephron development. The finding that hypoxia-mediated reduction of nephron numbers was progressively worsened into

the adulthood is intriguing and suggests that fetal hypoxia causes programming of aberrant kidney development and accelerates the aging process of the kidney during the postnatal development, which may contribute to an increased risk of cardiovascular disease, particularly hypertension in offspring. It has been shown that the aging process of the kidney is associated with reduced glomerular number, which contributes to an increased risk of hypertension and other cardiovascular diseases.

The Renin-Angiotensin System is a key regulator of blood pressure, fluid/electrolyte homeostasis, and kidney development (Kobori et al., 2006; Woods et al., 2001). A recent study demonstrated that prenatal nicotine exposure did not affect the expression of AT₁R in rat kidneys of 14-days, 30-days and 3-month offspring, as compared with the control groups²¹. However, the nicotine treatment decreased the expression of AT₂R in all the three post-natal ages. Here we reported that fetal hypoxia decreased the mRNA and protein expression of AT₁R just in adult offspring (5M) and the expression of AT₂R was not affected by this maternal stress suggesting that different maternal stress could affect the organogenesis of the kidney in different manners. Additionally, it has been shown that maternal protein restriction diet suppresses the expression of renal RAS probably due to the reduced number of glomeruli per kidney, which is associated with the increased arterial blood pressure in the adult rats (Woods et al., 2001).

It has been reported that AT₁R and AT₂R have a regulatory role in renal homodynamic and in tubular function, which is antagonist one form the other. Due to this, the balance between these two receptors can determine the renal status in health and disease. Although the expression of AT₁R and AT₂R alone is not a measure of kidney

function, a significant reduction of AT₁R/AT₂R protein density ratio in the kidney of adult offspring, whose mother were exposed to hypoxia during pregnancy, indicates that the renal function could be affected. Also these receptors coupled with ANG I-converting enzyme, which forms ANG II in the circulation are associated with the maintenance of blood pressure through renal and vascular mechanisms. Li et al (X. C. Li & Zhuo, 2008) demonstrated the intracellular expression of ANG II induces a significant increase in blood pressure that was associated with a higher expression of sodium hydrogen exchange transporter that is coupled with ANG II-AT₁ receptor axis. Using losartan, an antagonist for AT₁R, Woods et al. demonstrated that AT₁R played a key role in fetal programming of aberrant renal development and hypertension in offspring (Woods & Rasch, 1998). In the present study, we demonstrated that fetal hypoxia did not affect the expression of AT₁R in E21 and P7 rats, but significantly decreased AT₁R expression in adult offspring. Additionally, this effect is more pronounced in females than males. This is consistent with the finding of the greater reduction in nephron numbers in female than male adult offspring and suggests that the decreased AT₁R in adult kidneys is due to reduced nephron numbers in hypoxic-treated animals. This finding raise the possibility of a sex-dependent effect due to maternal hypoxia on the renal AT₁R expression might lead to sex differences on the impact of maternal hypoxia on adult disease. This could be evaluated in future studies. The finding that AT₂R in the kidney was not significantly affected by fetal hypoxia in all the developmental ages examined in the present study suggests a predominate location of AT₂R in extra-nephron tissues in the kidney. Indeed, Miyata *et al.* (Miyata, Park, Li, & Cowley, 1999) demonstrated that AT₁R, but not AT₂R, mRNA was detected in the nephron.

Although AT₁R plays an important role in normal kidney development (Oliverio et al., 1998), fetal hypoxia had no effect on AT₁R in the kidney but decreased nephron numbers in P7 rats, suggesting other mechanisms may be involved in the hypoxia-mediated abnormal nephron development. Possible mechanisms associated with the reduction of glomeruli that could be explored are the potential effects of fetal hypoxia on Wnt4 that is required by the metanephric mesenchyme for differentiation into nephron epithelia (Stark, Vainio, Vassileva, & McMahon, 1994), or fibroblast growth factor-2 (Karavanova, Dove, Resau, & Perantoni, 1996) and transforming growth factor β -2 (Sanford et al., 1997) that are capable of inducing nephrons remain an intriguing area for future investigation. Another possible mechanism to explore is to evaluate how prenatal hypoxia affects the expression of retinoid receptors, which mediates remodeling process in epithelial cells associated with nephron genesis (Batourina et al., 2001). Additionally, it has been shown that hypoxia increases the expression of glucocorticoid receptors in the primary human renal cortex epithelial cells (Jenq, Rabb, Wahe, & Ramirez, 1996), and that glucocorticoids play an important role in accelerating the maturation process of the kidney function (Petershack, Nagaraja, & Guillery, 1999; Seckl, 2004). Furthermore, the previous study demonstrated that hypoxia up-regulated the expression of angiopoietin-2 in cultured mouse kidney mesangial cells, which may have a synergistic paracrine role in the growth of glomerular endothelial during the development of the kidney (Yuan, Yang, & Woolf, 2000). In humans, it has been shown that the aging process of the kidney induces changes in anatomic and function of the renal system, including reduction of glomerular filtration rate, segmental glomerular sclerosis, reduction in the number of glomeruli and a decrease in renal mass (Epstein, 1996). These changes may result in

chronic kidney diseases a risk factor that eventually induced cardiac complications. Our results suggest that fetal hypoxia induces aberrant nephrogenesis and accelerates the aging process of the kidney. Additionally, the results suggest that the mechanisms other than the renal RAS may be involved in fetal hypoxia-mediated programming of abnormal kidney development. Future studies are required to further elucidate these unknown pathways.

References

- Barker, D. J. (1995). Fetal origins of coronary heart disease. *BMJ*, *311*(6998), 171-174.
- Barker, D. J. (1997). The fetal origins of coronary heart disease. *European heart journal*, *18*(6), 883-884.
- Batourina, E., Gim, S., Bello, N., Shy, M., Clagett-Dame, M., Srinivas, S., . . . Mendelsohn, C. (2001). Vitamin A controls epithelial/mesenchymal interactions through Ret expression. *Nature genetics*, *27*(1), 74-78. doi: 10.1038/83792
- Boubred, F., Buffat, C., Feuerstein, J. M., Daniel, L., Tsimaratos, M., Oliver, C., . . . Simeoni, U. (2007). Effects of early postnatal hypernutrition on nephron number and long-term renal function and structure in rats. *Am J Physiol Renal Physiol*, *293*(6), F1944-1949. doi: 00141.2007 [pii]
- 10.1152/ajprenal.00141.2007
- Brenner, B. M., & Chertow, G. M. (1994). Congenital oligonephropathy and the etiology of adult hypertension and progressive renal injury. *Am J Kidney Dis*, *23*(2), 171-175. doi: S027263869400020X [pii]
- Brenner, B. M., Garcia, D. L., & Anderson, S. (1988). Glomeruli and blood pressure. Less of one, more the other? *American journal of hypertension*, *1*(4 Pt 1), 335-347.
- Chen, J. H., Martin-Gronert, M. S., Tarry-Adkins, J., & Ozanne, S. E. (2009). Maternal protein restriction affects postnatal growth and the expression of key proteins involved in lifespan regulation in mice. *PloS one*, *4*(3), e4950. doi: 10.1371/journal.pone.0004950
- de Gasparo, M., Catt, K. J., Inagami, T., Wright, J. W., & Unger, T. (2000). International union of pharmacology. XXIII. The angiotensin II receptors. *Pharmacological reviews*, *52*(3), 415-472.
- Epstein, M. (1996). Aging and the kidney. *Journal of the American Society of Nephrology : JASN*, *7*(8), 1106-1122.
- Eriksson, J. G., Forsen, T., Tuomilehto, J., Winter, P. D., Osmond, C., & Barker, D. J. (1999). Catch-up growth in childhood and death from coronary heart disease: longitudinal study. *BMJ*, *318*(7181), 427-431.

- Gray, S. P., Denton, K. M., Cullen-McEwen, L., Bertram, J. F., & Moritz, K. M. (2010). Prenatal exposure to alcohol reduces nephron number and raises blood pressure in progeny. *Journal of the American Society of Nephrology : JASN*, *21*(11), 1891-1902. doi: 10.1681/ASN.2010040368
- Hostetter, T. H. (2003). Hyperfiltration and glomerulosclerosis. *Seminars in nephrology*, *23*(2), 194-199. doi: 10.1053/anep.2003.50017
- Jenq, W., Rabb, H., Wahe, M., & Ramirez, G. (1996). Hypoxic effects on the expression of mineralocorticoid and glucocorticoid receptors in human renal cortex epithelial cells. *Biochemical and biophysical research communications*, *218*(2), 444-448. doi: 10.1006/bbrc.1996.0079
- Julian, C. G., Vargas, E., Armaza, J. F., Wilson, M. J., Niermeyer, S., & Moore, L. G. (2007). High-altitude ancestry protects against hypoxia-associated reductions in fetal growth. *Archives of disease in childhood. Fetal and neonatal edition*, *92*(5), F372-377. doi: 10.1136/adc.2006.109579
- Karavanova, I. D., Dove, L. F., Resau, J. H., & Perantoni, A. O. (1996). Conditioned medium from a rat ureteric bud cell line in combination with bFGF induces complete differentiation of isolated metanephric mesenchyme. *Development*, *122*(12), 4159-4167.
- Keller, G., Zimmer, G., Mall, G., Ritz, E., & Amann, K. (2003). Nephron number in patients with primary hypertension. *The New England journal of medicine*, *348*(2), 101-108. doi: 10.1056/NEJMoa020549
- Kobori, H., Ozawa, Y., Suzaki, Y., Prieto-Carrasquero, M. C., Nishiyama, A., Shoji, T., . . . Navar, L. G. (2006). Young Scholars Award Lecture: Intratubular angiotensinogen in hypertension and kidney diseases. *American journal of hypertension*, *19*(5), 541-550. doi: 10.1016/j.amjhyper.2005.11.014
- Larsson, L., Aperia, A., & Wilton, P. (1980). Effect of normal development on compensatory renal growth. *Kidney international*, *18*(1), 29-35.
- Lawrence, J., Xiao, D., Xue, Q., Rejali, M., Yang, S., & Zhang, L. (2008). Prenatal nicotine exposure increases heart susceptibility to ischemia/reperfusion injury in adult offspring. *The Journal of pharmacology and experimental therapeutics*, *324*(1), 331-341. doi: 10.1124/jpet.107.132175
- Li, G., Xiao, Y., Estrella, J. L., Ducsay, C. A., Gilbert, R. D., & Zhang, L. (2003). Effect of fetal hypoxia on heart susceptibility to ischemia and reperfusion injury in the adult rat. *J Soc Gynecol Investig*, *10*(5), 265-274. doi: S1071557603000741 [pii]

- Li, X. C., & Zhuo, J. L. (2008). Intracellular ANG II directly induces in vitro transcription of TGF-beta1, MCP-1, and NHE-3 mRNAs in isolated rat renal cortical nuclei via activation of nuclear AT1a receptors. *American journal of physiology. Cell physiology*, 294(4), C1034-1045. doi: 10.1152/ajpcell.00432.2007
- Luzi, G., Bori, S., Iammarino, G., Taddei, F., Martines, G., Piatti, P., & Di Renzo, G. C. (1996). [Functional aspects of the fetal urinary apparatus in relation to growth]. *Archivio italiano di urologia, andrologia : organo ufficiale [di] Societa italiana di ecografia urologica e nefrologica / Associazione ricerche in urologia*, 68(5 Suppl), 9-12.
- Mao, C., Hou, J., Ge, J., Hu, Y., Ding, Y., Zhou, Y., . . . Zhang, L. (2010). Changes of renal AT1/AT2 receptors and structures in ovine fetuses following exposure to long-term hypoxia. *Am J Nephrol*, 31(2), 141-150. doi: 000259901 [pii]
- 10.1159/000259901
- Mao, C., Wu, J., Xiao, D., Lv, J., Ding, Y., Xu, Z., & Zhang, L. (2009). The effect of fetal and neonatal nicotine exposure on renal development of AT(1) and AT(2) receptors. *Reproductive toxicology*, 27(2), 149-154. doi: 10.1016/j.reprotox.2009.01.012
- Miyata, N., Park, F., Li, X. F., & Cowley, A. W., Jr. (1999). Distribution of angiotensin AT1 and AT2 receptor subtypes in the rat kidney. *The American journal of physiology*, 277(3 Pt 2), F437-446.
- Oliverio, M. I., Kim, H. S., Ito, M., Le, T., Audoly, L., Best, C. F., . . . Coffman, T. M. (1998). Reduced growth, abnormal kidney structure, and type 2 (AT2) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT1A and AT1B receptors for angiotensin II. *Proceedings of the National Academy of Sciences of the United States of America*, 95(26), 15496-15501.
- Petershack, J. A., Nagaraja, S. C., & Guillery, E. N. (1999). Role of glucocorticoids in the maturation of renal cortical Na⁺-K⁺-ATPase during fetal life in sheep. *The American journal of physiology*, 276(6 Pt 2), R1825-1832.
- Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., . . . Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development*, 124(13), 2659-2670.

- Seckl, J. R. (2004). Prenatal glucocorticoids and long-term programming. *European journal of endocrinology / European Federation of Endocrine Societies*, 151 Suppl 3, U49-62.
- Stark, K., Vainio, S., Vassileva, G., & McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature*, 372(6507), 679-683. doi: 10.1038/372679a0
- Tapanainen, P. J., Bang, P., Wilson, K., Unterman, T. G., Vreman, H. J., & Rosenfeld, R. G. (1994). Maternal hypoxia as a model for intrauterine growth retardation: effects on insulin-like growth factors and their binding proteins. *Pediatric research*, 36(2), 152-158.
- Tufro-McReddie, A., Romano, L. M., Harris, J. M., Ferder, L., & Gomez, R. A. (1995). Angiotensin II regulates nephrogenesis and renal vascular development. *The American journal of physiology*, 269(1 Pt 2), F110-115.
- Weinstein, J. R., & Anderson, S. (2010). The aging kidney: physiological changes. *Advances in chronic kidney disease*, 17(4), 302-307. doi: 10.1053/j.ackd.2010.05.002
- Woods, L. L., Ingelfinger, J. R., Nyengaard, J. R., & Rasch, R. (2001). Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatric research*, 49(4), 460-467.
- Woods, L. L., Ingelfinger, J. R., & Rasch, R. (2005). Modest maternal protein restriction fails to program adult hypertension in female rats. *American journal of physiology. Regulatory, integrative and comparative physiology*, 289(4), R1131-1136. doi: 10.1152/ajpregu.00037.2003
- Woods, L. L., & Rasch, R. (1998). Perinatal ANG II programs adult blood pressure, glomerular number, and renal function in rats. *The American journal of physiology*, 275(5 Pt 2), R1593-1599.
- Woods, L. L., Weeks, D. A., & Rasch, R. (2004). Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney international*, 65(4), 1339-1348. doi: 10.1111/j.1523-1755.2004.00511.x
- Yuan, H. T., Yang, S. P., & Woolf, A. S. (2000). Hypoxia up-regulates angiotensin-2, a Tie-2 ligand, in mouse mesangial cells. *Kidney international*, 58(5), 1912-1919. doi: 10.1111/j.1523-1755.2000.00363.x

Zhang, D. Y., Lumbers, E. R., Simonetta, G., Wu, J. J., Owens, J. A., Robinson, J. S., & McMillen, I. C. (2000). Effects of placental insufficiency on the ovine fetal renin-angiotensin system. *Experimental physiology*, 85(1), 79-84.

CHAPTER THREE

FETAL HYPOXIA INCREASES VULNERABILITY OF HYPOXIC-
ISCHEMIC BRAIN INJURY IN NEONATAL RATS: ROLE OF
GLUCOCORTICOID RECEPTORS

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Abstract

Gestational hypoxia is a common stress to the fetal development and increases the risk of neonatal morbidity. We tested the hypothesis that fetal hypoxia results in heightened brain vulnerability to hypoxic-ischemic injury in neonatal rats *via* down-regulation of glucocorticoid receptor in the developing brain. Time-dated pregnant rats were exposed to hypoxia (10.5% O₂) from day 15 to 21 of gestation. Brain HI injury was determined in day 10 pups. Maternal hypoxia resulted in asymmetric intrauterine growth restriction in the fetus. The brain HI injury was significantly increased in maternal hypoxia-treated pups as compared with the normoxia control in both males and females. Activation of brain GR by intracerebroventricular administration of dexamethasone produced a concentration-dependent reduction of HI-induced brain injury in control pups. Maternal hypoxia significantly decreased GR mRNA and protein abundance in the fetal brain and neonatal hippocampus and abolished the dexamethasone-mediated neuroprotective effect in pup brains. This was resulted from increased DNA methylation, decreased binding of transcription factors Egr-1 and Sp1 to GR gene exon 1₇ and 1₁₁ promoters, and reduced expression of GR exon 1₇ and 1₁₁ mRNA variants. The results demonstrate that gestational hypoxia causes epigenetic repression of GR gene expression in the developing brain resulting in the heightened brain vulnerability to HI injury in neonatal rats.

Introduction

Hypoxic-ischemic encephalopathy is the most common cause of brain damage due to systemic asphyxia, which may occur prior, during or after birth (Douglas-Escobar and Weiss, 2012). HIE occurs in about 2% of full-term infants and close to 60% in premature newborns, and causes significant mortality and lead to long-term neurologic sequelae, including learning disabilities, mental retardation, seizure and cerebral palsy (Graham et al., 2008; Kurinczuk et al., 2010). Although little is known about the pathogenesis of HIE, recent studies have suggested that adverse intrauterine environment may contribute to aberrant brain development (Tomalski and Johnson, 2010; Li et al., 2012). Hypoxia is a common form of intrauterine stress, and the fetus may experience prolonged hypoxic stress under a variety of conditions, including pregnancy at high altitude, pregnancy with anemia, placental insufficiency, cord compression, preeclampsia, heart, lung and kidney disease, or with hemoglobinopathy. Although it has been shown that fetal hypoxia affects normal brain development and induces neurological deficit in a variety of behavioral tests in offspring (Li et al., 2012), the effect of fetal hypoxia-mediated stress on newborn brain HIE remains elusive.

Many factors may be involved in the stress response in the developing brain. Among them, glucocorticoids are essential for the brain development and play a center role in the response to stress. The effects of glucocorticoids are mainly mediated *via* binding to glucocorticoid receptors, and GRs are highly expressed in the developing brain with dynamic and complicated ontogeny. It has been demonstrated in humans and rodents that early life environment and events are critical in programming tissue-specific GR expression levels, particularly in the hippocampus (Xiong and Zhang; Weaver et al., 2005; Weaver et al., 2007; Mueller and Bale, 2008; Oberlander et al., 2008; Turner et al.,

2008; Turner et al., 2010; Li et al., 2012). Both neurodegenerative and neuroprotective effects of glucocorticoids have been reported (Abraham et al., 2001). Glucocorticoids have been shown to affect the vulnerability of fetal and neonatal brains to hypoxia-ischemia challenge; however, the results were inconsistent and dependent on experimental protocol, dosage, timing, animal age, strains and species (Tombaugh et al., 1992; Kauffman et al., 1994; Tuor, 1995; Flavin, 1996; Tuor, 1997; Whitelaw and Thoresen, 2000). It appears that the concentration and duration of glucocorticoid treatment are the two key factors determining either detrimental or beneficial effects of glucocorticoids in the brain. Although exposure to long-term and high levels of glucocorticoids enhances neurotoxic effects in brain injury, physiological or slightly supra-physiological levels of glucocorticoids confer the brain protective effect to HIE challenges (Abraham et al., 2001). Herein, we present evidence of a novel finding that chronic fetal hypoxia down-regulates GR expression in the developing brain resulting in the increased brain susceptibility to HI injury in neonatal rats, and suggest new insights of molecular mechanisms linking fetal hypoxia to the heightened HIE vulnerability in newborns.

Materials and Methods

Experimental Animals

Pregnant Sprague Dawley rats were randomly divided into 2 groups: normoxic control and hypoxic treatment of 10.5% O₂ from day 15 to 21 of gestation, as described previously (Patterson et al., 2010). Given that hypoxia decreased maternal food intake by approximately 40%, a group of pregnant rats were randomized to 60% of control food intake under the normoxic condition during the same gestational period as a pair-fed

control. On day 21 of pregnancy, some rats were euthanized and fetal brains were isolated. Other rats were allowed to give birth, and further studies were conducted in 10-day-old neonatal pups of both sexes. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Brain HI Treatment

Brain HI injury was induced in P10 pups, as previously described (Li et al., 2012). Pups were anesthetized with 2% isoflurane and the right common carotid artery was ligated. After recovery for 1 hour, pups were treated with 8% O₂ for 1.5 hours. To determine the role of GR in brain HI injury, GR agonist dexamethasone (Sigma-Aldrich) was injected into the right lateral ventricle prior to the HI treatment. Pups were anesthetized and fixed on a stereotaxic apparatus (Stoelting, Wood Dale, IL). An incision was made on the skull surface and bregma was exposed. Dexamethasone was injected at a rate of 1 μ l/minute with a 10 μ l syringe (Stoelting, Wood Dale, IL) on the right hemisphere following the coordinates relative to bregma: 2 mm posterior, 1.5 mm lateral and 3.0 mm below the skull surface (Li et al., 2012). Saline was injected as the vehicle control. The injection lasted 2 minutes and the needle was kept for additional 5 minutes before its removal. The incision was sutured.

Measurement of Brain Infarct Size

Forty-eight hours after the HI treatment, pups were sacrificed and brain infarct

size was determined as previously described (Li et al., 2012). Briefly, coronal slices of the brain (2 mm thick) were cut and immersed in a 2% solution of 2,3,5-triphenyltetrazolium chloride monohydrate (Sigma-Aldrich) for 5 minutes at 37°C and then fixed by 10% formaldehyde overnight. Each slice was weighed, photographed separately and the percentage of infarction area for each slice was analyzed by Image J software (Version 1.40; National Institute of Health, Bethesda, MD), corrected by slice weight, summed for each brain, and expressed as a percentage of whole brain weight.

Western Blot

GR protein abundance was determined by Western blot (Xue et al., 2011). Briefly, brains were homogenized and protein concentrations in supernatants were determined. Samples with equal amounts of proteins were separated by electrophoresis, and were probed with primary antibodies against GR or HIF 1 α (Santa Cruz Biotechnology). Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody, and proteins were visualized with enhanced chemiluminescence reagents. The target protein abundance was normalized to actin.

Real-time RT-PCR

Total RNA was isolated and subjected to reverse transcription with Superscript III First-Strand Synthesis System (Invitrogen). The abundance of GR mRNA and the alternate exon 1 variants was measured with real-time PCR using iQ SYBR Green Supermix (Bio-Rad), as described previously (Meyer et al., 2009; Xiong et al., 2012). Primers used were listed in Table 1. Real-time PCR was performed in a final volume of

25 µl and each PCR reaction mixture consisted of 500 nM of primers and iQ SYBR Green Supermix containing 0.625 unit hot-start Taq polymerase, 400 µM each of dATP, dCTP, dGTP, and dTTP, 100 mM KCl, 16.6 mM ammonium sulfate, 40 mM Tris-HCl, 6 mM MgSO₄, SYBR Green I, 20 nM fluorescing and stabilizers. We used the following real time-PCR protocol: 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 seconds, annealing for 10 seconds at appropriate temperature depending on the primer sequence, 72°C for 10 seconds. Serial dilutions of the positive control were done on each plate to create a standard curve for the quantification. PCR was done in triplicate and threshold cycle numbers were averaged for each sample.

Methylated DNA Immunoprecipitation

MeDIP assays were performed with the MeDIP kit (Active Motif, Carlsbad, CA, USA), following the manufacturer's instruction. Briefly, genomic DNA was extracted from tissues and sonicated to yield fragments ranging in size from 200 to 600 base pairs. The double strand DNA fragments were denatured at 95 °C to produce single strand DNA, and a 5-methylcytosine (5-mC) antibody was then used to precipitate DNA containing 5-mC. The 5-mC enriched DNA was subjected to PCR analysis with primers flanking the GR promoter and the PCR products were visualized with 3% agarose gel stained with ethidium bromide and analyzed with ImageJ software. Two sets of primers flanking transcription factor binding sites SP1 and Egr-1 at exon 17 promoter and SP1 at exon 11 promoter were used: 5'-agcccctctgctagtgtgac-3' (Promoter-17-F), 5'-ttctcttctcccaggctcc-3' (Promoter-17-R); 5'-agtctggcgtccttttgg-3' (Promoter-11-F), 5'-ccaatccaccctacaagccc-3' (Promoter-11-R).

Table 1. Primer sequences for GR and GR gene first exons.

	Forward	Reverse
exon 1 ₄	AAGCAACACCGTAACACCTT	AGAAGCAGCAGCCACTGA
exon 1 ₅	CATGCAACTTCCTCCGAGT	
exon 1 ₇	GGAGCCTGGGAGAAGAGAAA	
exon 1 ₁₁	GCCGCAGAGAACTCAACAG	
exon 1 ₁₀	CACGCCGACTTGTTTATC	TCTGCTGCTTGGAATCTG
exon 1 ₆	ACCTGG CGG CAC GCG AGT	GCAGCCACTGAGGGCGAAGA
exon 1 ₈	GACAGTCGCCAACAGGTAA	TGAGAAGCAGCAGCCACT
exon 1 ₉	GTCAGTGCCTGGAGCCCGAG	AGCAGCCACTGAGGGCGAAG
GR	AGGTCTGAAGAGCCAAGAGTTA	TGGAAGCAGTAGGTAAGGAGAT
actin	TCAGGTCATCACTATCGGCAAT	ACTGTGTTGGCATAGAGGTCTT

Chromatin Immunoprecipitation

ChIP assay was performed using the Chip-IT Express Kit (Active Motif), as described previously. Briefly, tissues were minced and fixed with 1% formaldehyde to crosslink and maintain the DNA/protein interactions. After the reactions were stopped with glycine, tissues were washed with PBS. Chromatin extracts were sonicated to produce DNA fragments between 200 and 600 base pairs. Antibody against Sp1, MeCP2 (Active Motif), Egr-1 (Santa Cruz Biotechnology), and MBD1 (Abcam) were incubated with the chromatin extracts to precipitate the transcription factor/DNA complexes. Crosslinking was then reversed using a salt solution and proteins were digested with proteinase K. The antibody-pulled chromatin extracts were subjected to PCR assay using two sets of primers that flank the predicted transcription factor binding sites at GR exon 1₇ and exon 1₁₁ promoters, as described above in MeDIP. PCR products were visualized by 3% agarose Gel stained with ethidium bromide and analyzed with ImageJ software.

Statistical Analysis

Data are expressed as mean \pm SEM. Experimental number (n) represents fetuses and neonates from different dams. Statistical significance ($P < 0.05$) was determined by analysis of variance followed by Neuman-Keuls post hoc testing or Student's *t* test, where appropriate.

Results

Maternal Hypoxia Induced Asymmetrical Growth Restriction in the Fetus

There was no significant difference in the litter size between normoxia control

(10.0 ± 1.6) and hypoxic (10.7 ± 0.8) groups. Maternal hypoxia resulted in a significant decrease in body and brain weight in E21 fetuses, but a significant increase in the brain to body weight ratio (Figure 1). In P10 pups, no significant differences in body and brain weight were observed between control and hypoxic animals, suggesting a catch-up growth of hypoxic neonates.

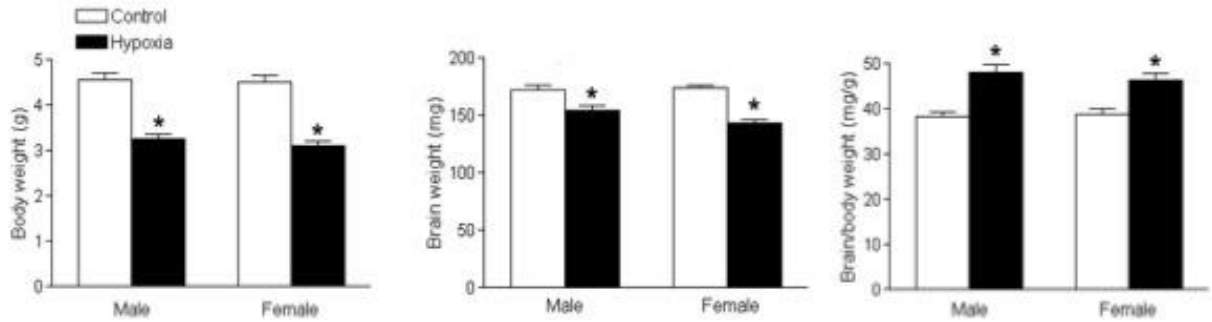
Maternal Hypoxia Increased Neonatal Brain Vulnerability to HI Injury

Maternal hypoxia significantly increased protein abundance of HIF-1 α in both, the fetal and P10 pup brain (Figure 2), indicating hypoxia in the fetal and P10 pup brain. To evaluate the effect of fetal hypoxia on brain lesion vulnerability, pups from normoxic and hypoxic animals were induced to brain lesion by HI at P10. Prenatal hypoxia significantly increased HI-induced brain infarct size in both male and female pups (Figure 3). To verify that this effect was not due to decreased maternal food intake by hypoxia, a pair-fed study of approximately 60% maternal food intake as that observed in hypoxic pregnant rats was conducted. No significant differences were observed in HI-induced brain infarct size in P10 pups by maternal food restriction (Figure 4).

Dexamethasone Reduced HI-Induced Neonatal Brain Damage

The neuroprotective effect of dexamethasone on HI-induced neonatal brain damage was examined *via* intracerebroventricular administration of dexamethasone prior to the HI treatment. Figure 5 shows a concentration-dependent neuroprotective effect of dexamethasone on HI-induced brain injury in both male and female pups.

A



B

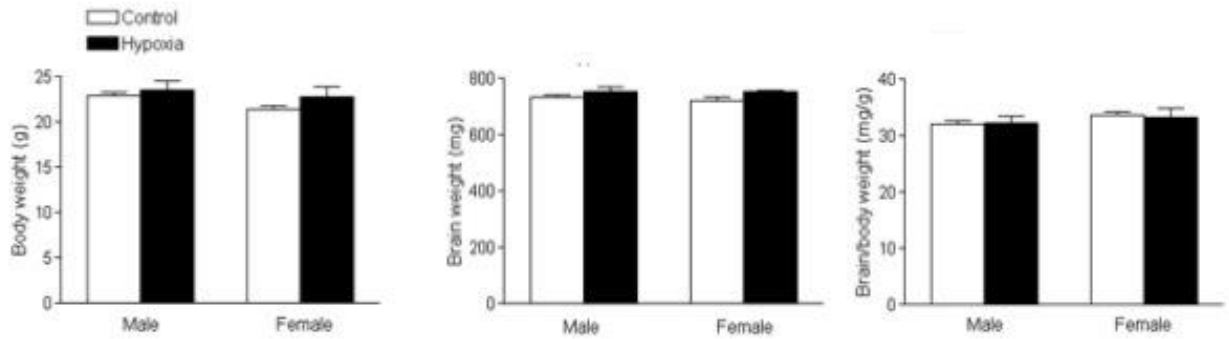


Figure 1. Effect of maternal hypoxia on fetal (A) and P10 pups (B) body and brain weight. Pregnant rats were exposed to hypoxia during their last third of gestational period. Body and brain weight were measured for fetus and P10 neonates and the brain/body weight ratio was reported. Four groups were evaluated for each stage. For fetus stage: male control ($n=12$), male hypoxia ($n=12$) female control ($n=13$) and female hypoxia ($n=14$). For the P10 pups: male control ($n=12$), male hypoxia ($n=9$) female control ($n=9$) and female hypoxia ($n=7$) Data are mean \pm SEM. * $P < 0.05$, hypoxia versus control. (P10 – postnatal day 10)

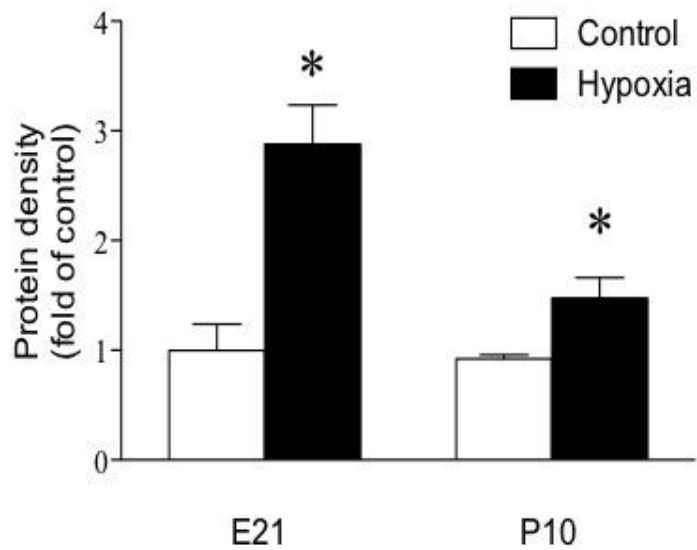


Figure 2. Effect of maternal hypoxia on HIF-1 α protein abundance in fetal and P10 pups brains. Brains were isolated from 21-day fetuses and P10 neonates from pregnant rats treated with normoxia control or hypoxia from day 15 to day 21 of gestation. HIF-1 α protein abundance was determined using Western blot analysis. Actin was used as a loading control. Data are mean \pm SEM, $n = 5$. * $P < 0.05$, hypoxia versus control. (E21 – 21-day fetus; P10 – postnatal day 10)

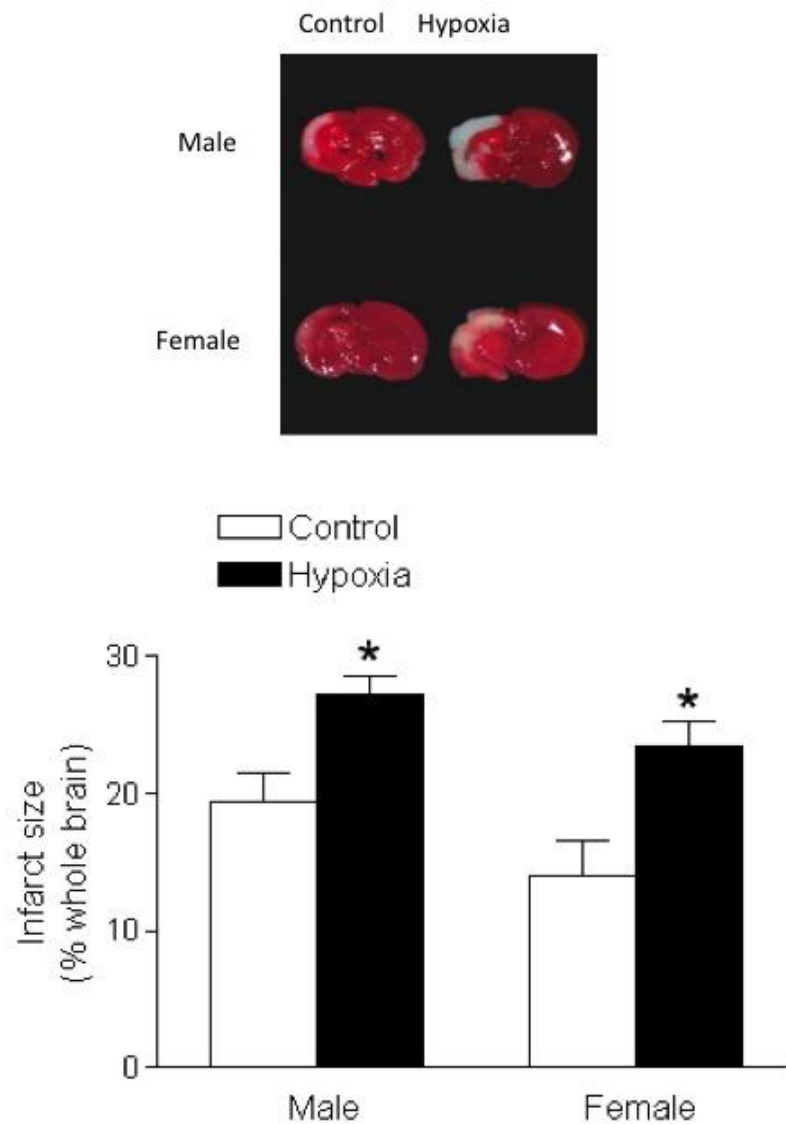


Figure 3. Effect of maternal hypoxia on HI-induced brain infarct size in P10 pups. Pregnant rats were exposed to hypoxia during their last third of gestational period. Brain injury was induced by carotid common occlusion on P10 neonates followed by hypoxia treatment. Infarct size was measured and reported as percent of the whole brain. Four groups were evaluated: male control ($n=8$), male hypoxia ($n=20$) female control ($n=11$) and female hypoxia ($n=18$). Data are mean \pm SEM, $n = 8-20$. * $P<0.05$, hypoxia versus control. (HI – Hypoxic Ischemic; P10 – postnatal day 10)

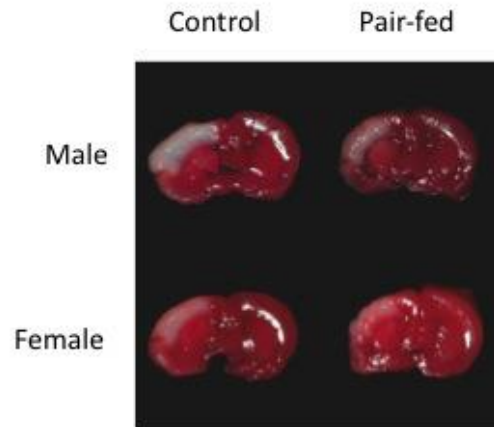


Figure 4. Effect of maternal food restriction (Pair-Fed) on HI-induced brain infarct size in P10 pups. Pregnant rats were provided with an amount of food similar to the food intake reported by hypoxic pregnant rats. Brain injury was induced on offspring P10 neonates and infarct size was measured and reported as percent of the whole brain. Data are mean \pm SEM. $n = 4$. (HI – Hypoxic Ischemic; P10 – postnatal day 10)

Maternal Hypoxia Down-Regulated GR Expression in the Developing Brain

The effect of maternal hypoxia on GR protein and mRNA abundance in the developing brain was shown in figure 6. Maternal hypoxia resulted in a significant decrease in protein and mRNA abundance of GR in brains of both female and male E21 fetuses (Figure 6A). In P10 neonates, maternal hypoxia significantly reduced GR expression in the hippocampus (Figure 6B).

Maternal Hypoxia Selectively Decreased GR Alternative Exon 1 mRNA Variants

GR gene has multiple alternative first exons that play important roles in regulating tissue specific expression of GR, and each exon 1 has its own promoter (Xiong and Zhang). Among the multiple exon 1s, exon 1₄ to 1₁₁ are located in the proximal region ranged from about -4.2 to -1.7 Kb upstream of exon 2, and exhibit substantial promoter activities. All exon 1 variants have unique splice donor sites and share a common exon 2 splice acceptor site, encoding a common protein. To investigate the effect of fetal hypoxia on the levels of specific GR exon 1 transcripts, quantitative RT-PCR assays were carried out with primers designed to amplify specific mRNA transcripts containing GR exon 1₄ to exon 1₁₁. As shown in figure 7A, in brains of both male and female fetuses, the abundance of exon 1₇ and 1₁₁ containing transcripts was significantly decreased by the hypoxic treatment. Other GR exon 1 mRNA variants were not changed by hypoxia. Similar reductions of exon 1₇ and 1₁₁ transcripts were also demonstrated in the hippocampus of P10 neonates (Figure 7B).

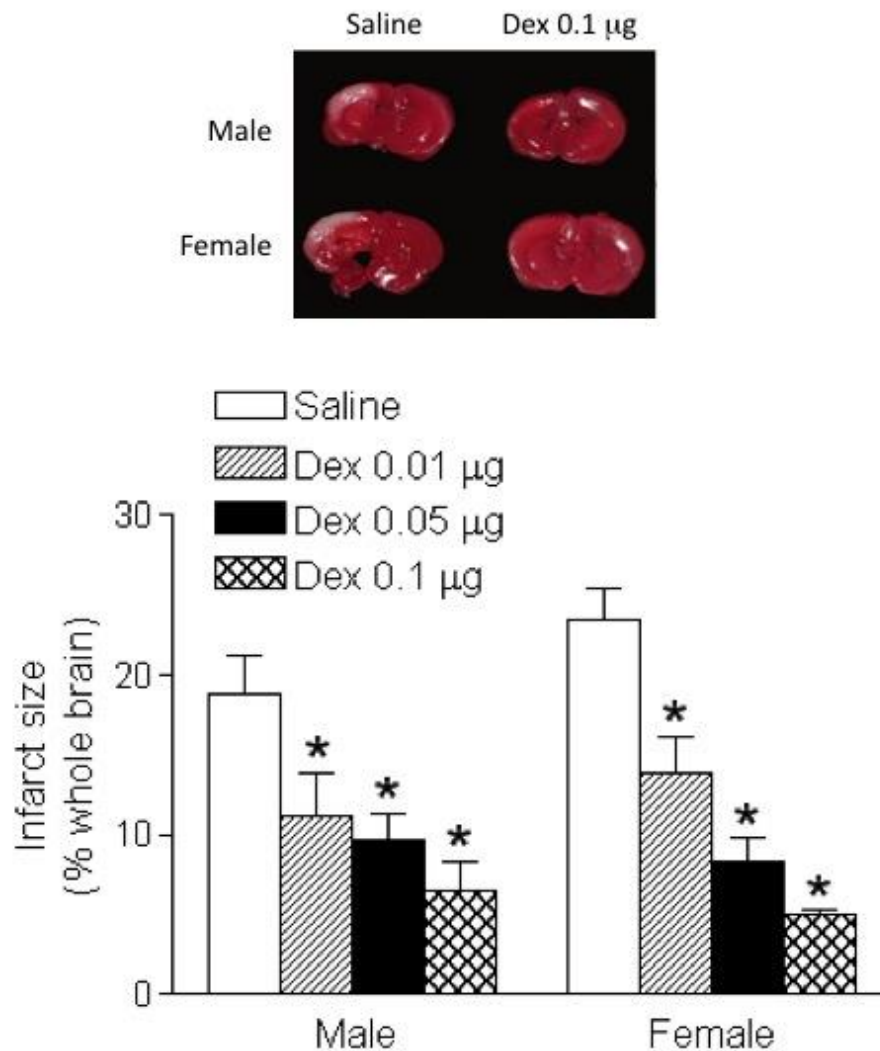


Figure 5. Effect of dexamethasone on HI-induced brain infarct size in P10 pups. Three groups of Sprague Dawley P10 pups were injected with dexamethasone by ICV injections, each group with a different dose. Brain injury was induced on each pup and infarct size was measured and reported. Control group received a saline injection. Eight groups were evaluated: control (male: $n=4$; female: $n=6$), 0.01ug dexamethasone (male: $n=10$; female: $n=10$), 0.05ug dexamethasone (male: $n=12$; female: $n=9$) and 0.1ug dexamethasone (male: $n=11$; female: $n=12$) Data are mean \pm SEM. * $P<0.05$,

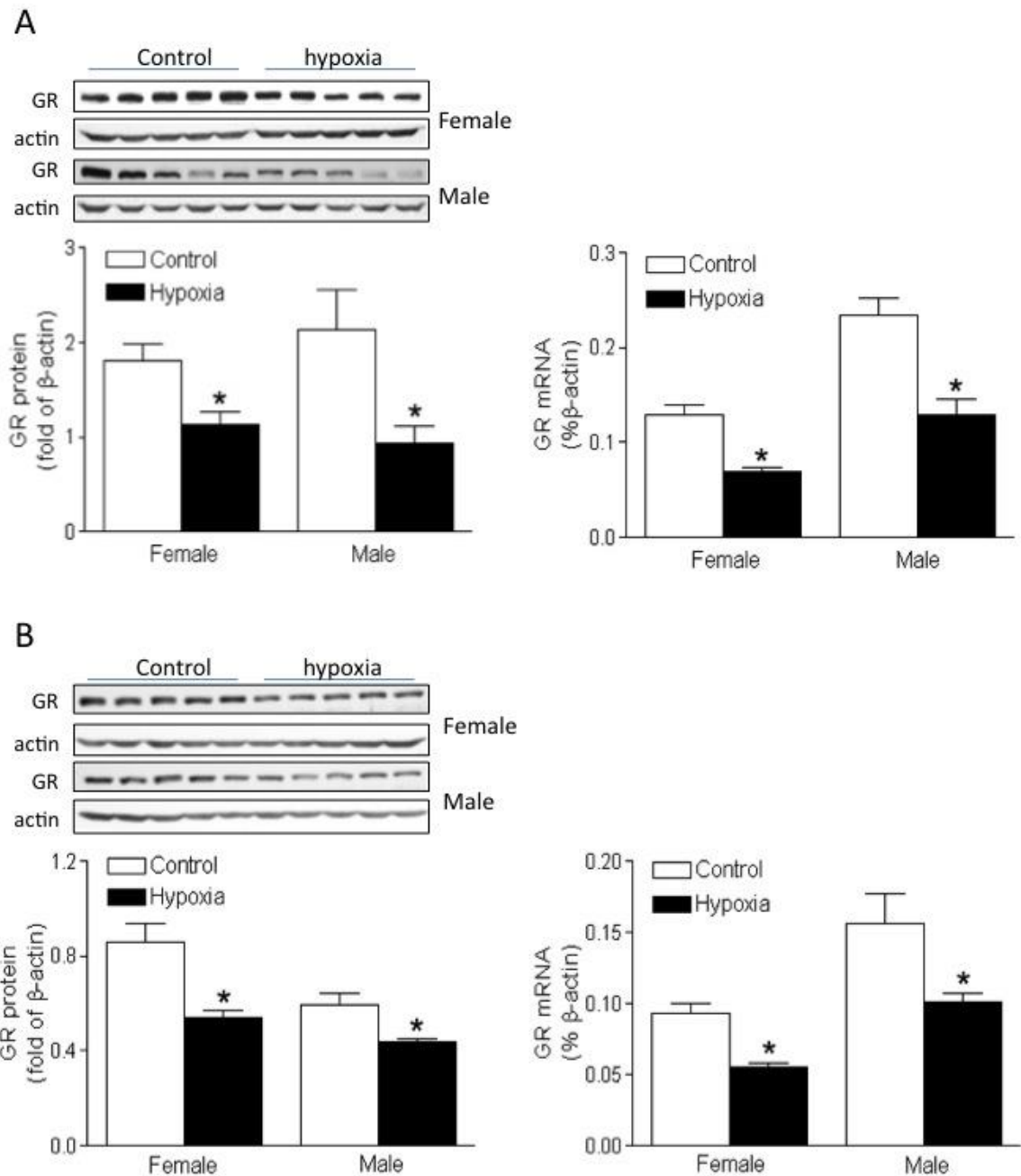


Figure 6. Effect of maternal hypoxia on glucocorticoid receptor (GR) protein and mRNA abundance in the fetal brain (A) and neonatal hippocampus (B). Brains were isolated from 21-day fetuses and P10 offspring rats from pregnant rats treated with normoxia control or hypoxia from day 15 to day 21 of gestation. GR protein and mRNA abundance was determined using Western blot analysis and quantitative real-time RT-PCR, respectively. Actin was used as a loading control. Data are mean \pm SEM, $n = 5$. * $P < 0.05$.

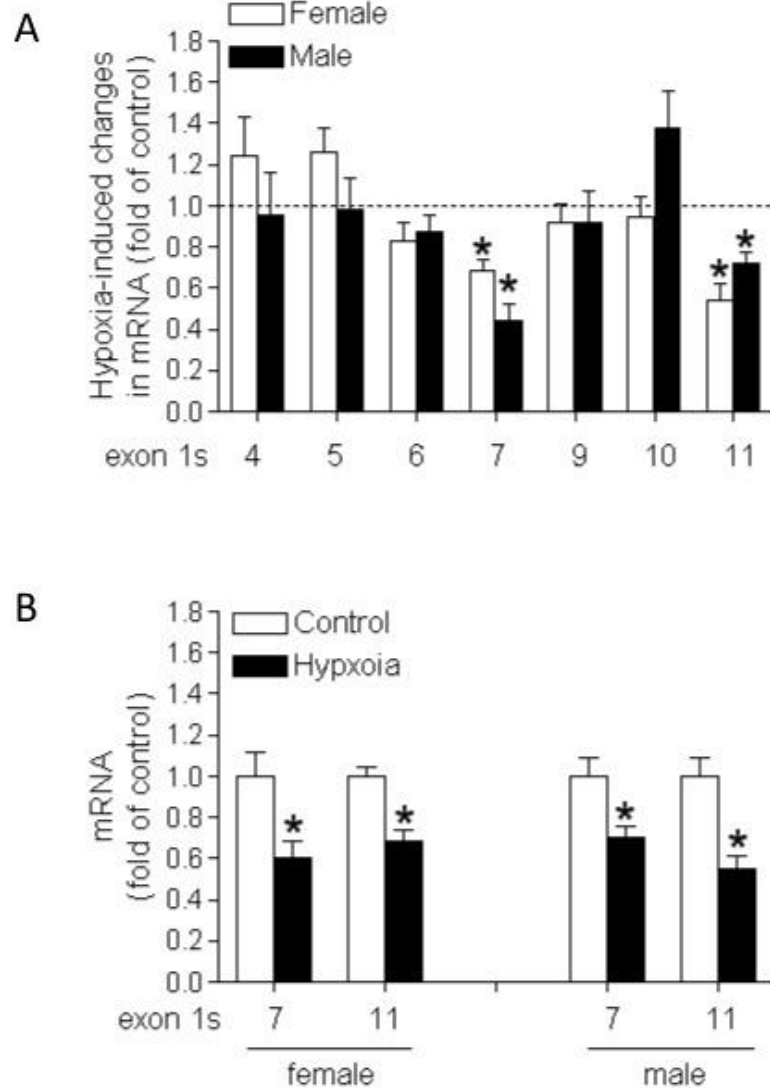
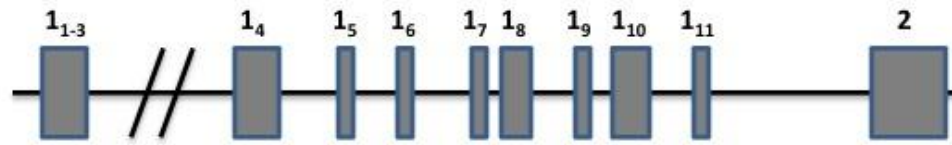


Figure 7. Effect of maternal hypoxia on mRNA abundance of glucocorticoid receptor (GR) exon 1 variants in the fetal brain (A) and neonatal hippocampus (B). Brains were isolated from 21-day fetuses and hippocampi were isolated from P10 pups offspring of control and prenatally hypoxic animals. GR exon-1 variants mRNA abundance was determined by quantitative real-time RT-PCR. Data are mean \pm SEM, $n = 5$. * $P < 0.05$, hypoxia versus control. (P10 – postnatal day 10)

Maternal Hypoxia Increased Methylation of GR 1₇ and 1₁₁ Promoters

Accumulating evidence indicates that prenatal stress may affect GR gene expression through epigenetic modification of promoter methylation (Mulligan et al., 2012). The effects of maternal hypoxia on DNA methylation of GR exon 1₇ and 1₁₁ promoters were determined by precipitation of methylated DNA and subsequent PCR analysis. Promoter methylation of GR exons 1₇ and 1₁₁ in the fetal brain was significantly increased by maternal hypoxia (Figure 8).

Maternal Hypoxia Reduced Transcription Factor Binding to GR Exon 1₇ and 1₁₁ Promoters

To determine the functional significance of increased promoter methylation, the binding of methyl-CpG binding proteins MeCP2 and MBD1, as well as transcription factors Sp1 and Egr-1 to GR exon 1₇ and 1₁₁ promoters was determined in the fetal brain by ChIP assays. As shown in figure 9, maternal hypoxia significantly increased the binding of MeCP2 to both GR exon 1₇ and 1₁₁ promoters, but had no effect on the binding of MBD1 to the GR promoters. On the other hand, maternal hypoxia significantly decreased the binding of Egr-1 to exon 1₇ promoter and Sp1 to exon 1₁₁ promoter, respectively, in the fetal brain (Figure 9).

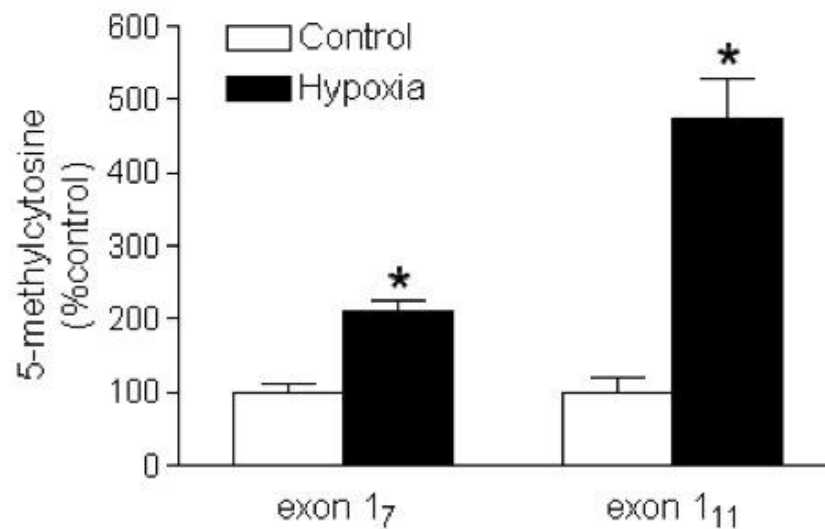


Figure 8. Effect of maternal hypoxia on promoter methylation of GR exon 1₇ and 1₁₁ in the fetal brain. Brains were isolated from 21-day fetuses from pregnant rats treated with normoxia control or hypoxia at 10.5% oxygen level from day 15 to day 21 of gestation. Methylation of promoter region, of GR exon 1 variants, was determined by Methylated DNA Immunoprecipitation Data are mean \pm SEM, $n = 5$. * $P < 0.05$, hypoxia versus control.

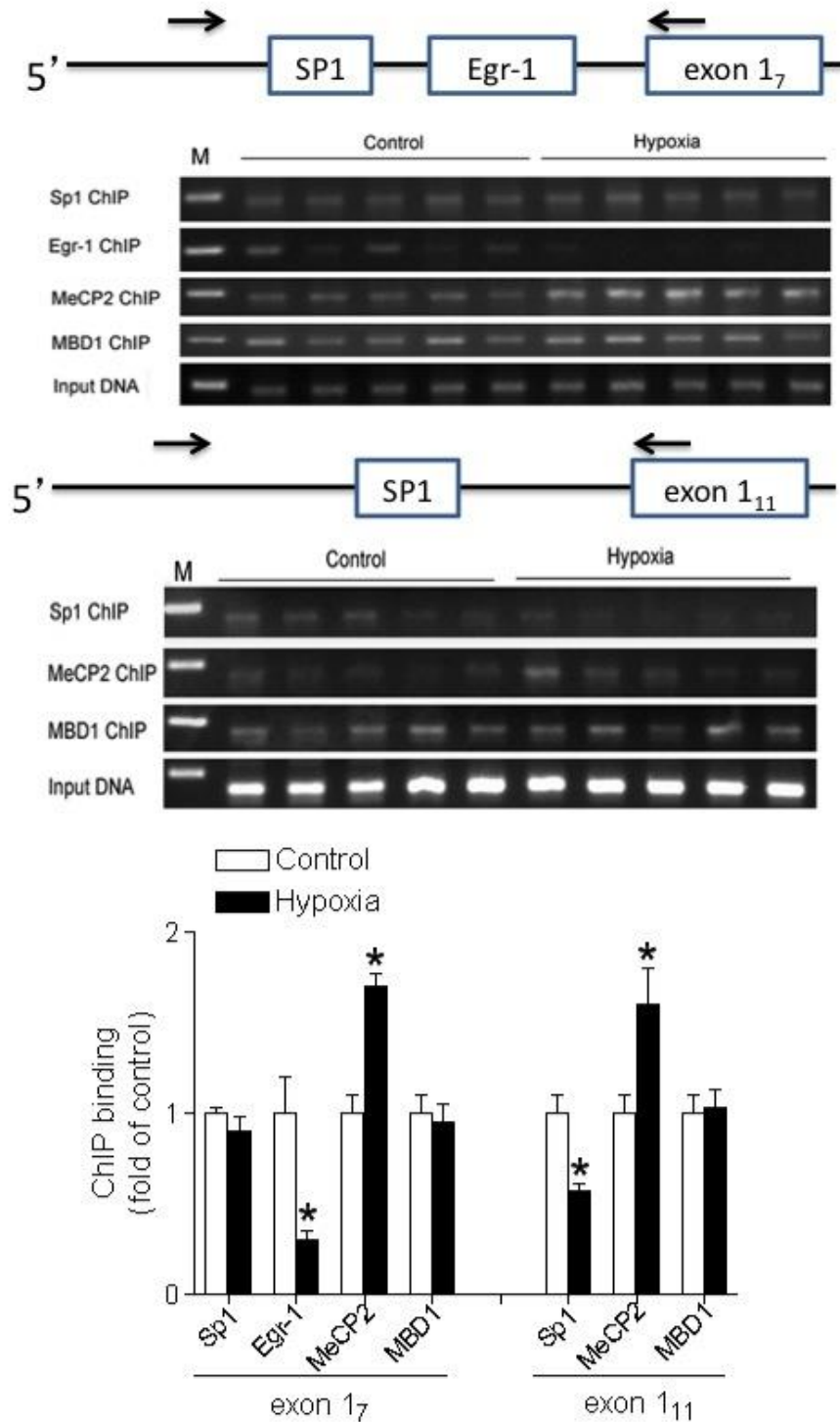


Figure 9. Effect of maternal hypoxia on binding of methyl-binding proteins and transcription factors to GR exon 1₇ and 1₁₁ promoters in the fetal brain. Brains were isolated from 21-days fetuses of control and prenatally hypoxic animals. Binding of transcription factors specificity protein 1 (Sp1) and early growth response protein 1 (Egr-1), and methyl CpG binding protein 2 (MeCP2) and methyl CpG binding domain protein 1 (MBD1) was determined by ChIP assays. M, DNA markers. Data are mean \pm SEM, $n = 5$. * $P < 0.05$, hypoxia versus control.

Maternal Hypoxia Abrogated the Neuroprotective Effect of Dexamethasone

The functional significance of fetal hypoxia-induced down-regulation of GR in the developing brain was determined by evaluating dexamethasone-mediated neuroprotective effect of HI-induced neonatal brain injury in the hypoxia-treated animals. As shown in figure 10, maternal hypoxia abolished dexamethasone-induced neuroprotective effect of HI-induced neonatal brain injury in both male and female pups.

Discussion

The present study demonstrates for the first time that fetal hypoxia increases the vulnerability of neonatal brains to hypoxic-ischemic encephalopathy in a rat model. Intracerebroventricular administration of a synthetic glucocorticoid dexamethasone protects the neonatal brain from hypoxic-ischemic brain injury, suggesting a local and direct neuroprotective effect of GR activation in the neonatal brain. Fetal hypoxia significantly down-regulates GR expression in the developing brain and abrogates the GR-mediated neuroprotection in the neonatal brain.

The finding that fetal hypoxia and asymmetrical growth restriction increased the vulnerability of neonatal rat brains to HI-induced injury suggests a novel mechanism in the understanding of pathogenesis of HIE that occurs at a significantly higher rate in compromised pregnancy and fetal development. The stage of brain development in P10 neonatal rats is approximately equivalent to that in full-term human baby (Rice et al., 1981; Towfighi et al., 1991). The approach of the Vannucci model in inducing HI injury in P10 rat brains provided a means of evaluating potential brain injury caused by birth asphyxia in full-term newborns. This model consists of unilateral common carotid artery

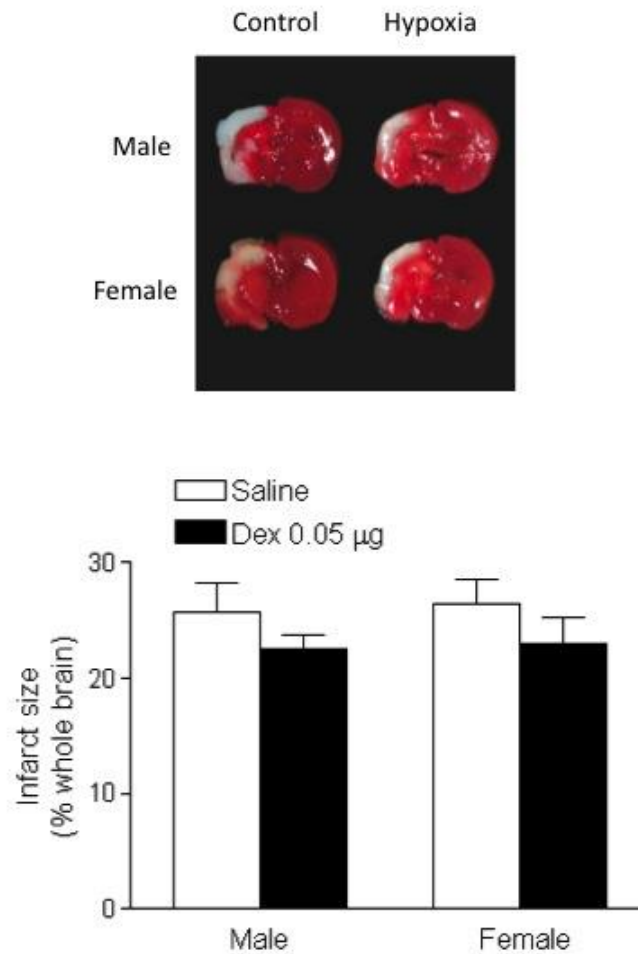


Figure 10. Effect of maternal hypoxia on dexamethasone-mediated neuroprotective effect in P10 pups. Brains were isolated from P10 offspring from pregnant rats treated with normoxia control or hypoxia at 10.5% oxygen level from day 15 to day 21 of gestation. ICV injections of dexamethasone were applied on the right brain hemisphere before the induced brain injury by common carotid occlusion was assessed, followed by hypoxia treatment. Infarct size was measured and reported as percent of the whole brain. Four groups were evaluated: male control ($n=5$), male dexamethasone ($n=5$), female control ($n=10$) and female dexamethasone ($n=7$). Data are mean \pm SEM

ligation followed by systemic hypoxia produced by the inhalation of 8% oxygen (Vannucci and Vannucci, 2005). Although there is a large body of evidence indicating a close link between fetal stress and an increased risk of cardiovascular and metabolic syndrome, stroke, and neurobehavioral pathogenesis in adolescence and/or adulthood, little is known about the effect of fetal stress on the susceptibility of neonatal HIE. Consistent with the present finding, a recent study demonstrated that maternal nicotine administration resulted in the increased vulnerability of HI-induced brain injury in neonatal rats (Li et al., 2012). Thus, these findings provide new insights linking fetal stress and pathophysiological consequences of heightened HIE in newborns. The lack of effect of maternal food restriction in the pair-fed study suggests a hypoxia-specific effect on the vulnerability of HI-induced brain injury in neonatal rats.

Although the mechanisms underlying fetal stress-induced increase in neonate brain HI injury remain largely elusive, the present study demonstrated that intracerebroventricular administration of dexamethasone produced a concentration-dependent decrease of HI-induced brain injury in both male and female neonatal rats, suggesting a neuroprotective effect of local and direct activation of GR in neonatal brains. Dexamethasone is a synthetic glucocorticoid that provides protection against hypoxic ischemic brain damage (Tuor et al., 1997). The present finding is consistent with the previous studies showing a dose-dependent inhibition of hypoxic-ischemic brain injury in neonatal rats by dexamethasone (Barks et al., 1991; Feng et al., 2011). More importantly, the present study demonstrated that fetal hypoxia abrogated the dexamethasone-induced neuroprotective effect in the neonatal brain, suggesting a possible mechanism of GR in fetal stress-mediated vulnerability of HIE in the neonate.

The loss of dexamethasone-mediated neuroprotection in the neonate in hypoxic animals is likely due to the down-regulation of GR expression in the developing brain. The GR activity in regulating tissue and cell function relies on a tight control of its expression levels. In both humans and rodents the regulation of GR levels occurs almost exclusively at the transcriptional level. Early-life environmental events play a key role in programming of tissue GR levels (O'Donnell et al., 1994; Liu et al., 1997; Bertram et al., 2001; Weaver et al., 2004). In the present study, GR protein and mRNA abundance in fetal and neonatal brains was significantly decreased by fetal hypoxia. This is consistent with the previous finding that fetal hypoxia resulted in a significant decrease in GR expression in fetal and neonatal hearts (Xue et al., 2011). These findings suggest that the down-regulation of GR may be a common response to fetal stress among tissues. Human and rat GR genes show a high similarity and both have 11 untranslated exon 1s, all of which have unique splice donor sites and share a common exon 2 splice acceptor site, encoding a common protein (McCormick et al., 2000; Turner and Muller, 2005; Turner et al., 2006). Similar to human GR gene, in rats, 8 of the alternate exon 1s, 1₄ to 1₁₁, are located in the proximal region upstream of exon 2, and exhibit substantial promoter activities (McCormick et al., 2000). Whereas the mechanisms regulating the differential usage of these first exons in different tissues and individuals, and the role of the 5' untranslated region in splicing of the coding exons remain poorly understood, multiple first exons plus modulation of alternative first exon activity by their promoters represent the complexity orchestrated by tissue-specific transcription factors in programming of GR expression patterns in a tissue-specific manner. In the present study, we found that fetal hypoxia resulted in a significant decrease in expression levels of GR mRNA variants

containing exon 1₇ and 1₁₁ in fetal brains, which sustained in the hippocampus of P10 neonatal brains. Whereas exon 1₇ has been shown to play an important role in regulating hippocampus GR expression and in “resetting” the HPA axis sensitivity resulting from maternal care and neonatal handling (McCormick et al., 2000; Oberlander et al., 2008), fetal stress-mediated modulation of exon 1₁₁ expression in regulating GR expression in the brain has not been reported. Indeed, a previous study suggested that neonatal handling had no significant effect on the expression of exon 1₁₁ in rat hippocampus (McCormick et al., 2000). Thus, the present finding of fetal hypoxia-induced down-regulation of exon 1₁₁ demonstrates the complexity of GR exon 1s involved in stress-specific and developmental age-specific regulation of GR expression in the developing brain, and suggests a novel mechanism of exon 1₁₁ in regulating brain GR expression patterns caused by fetal hypoxia.

The finding that fetal hypoxia-induced brain GR down-regulation sustained in the P10 neonate suggests a permanent GR repression in the developing brain. Accumulating evidence indicates that perinatal stress affects GR gene expression mainly through epigenetic mechanisms, and increased methylation of GR promoters has been associated with early life experiences in both humans and rats (Oberlander et al., 2008; Suderman et al., 2012). As important regulators of chromosome structure and gene expression, the methyl-CpG binding proteins, MeCP2 and MBD1 can bind to 5' methylated cytosine of CpGs on DNA and suppress gene expression (Guy et al., 2011). The present study showed the binding of both MeCP2 and MBD1 to the promoters of GR exon 1₇ and 1₁₁ in the fetal brain, and the binding of MeCP2 was significantly increased by fetal hypoxia. Increased methyl-CpG binding proteins to GR promoters is likely to induce chromatin

remodeling that make local chromatin structure more condensed and less accessible to transcription factor binding (Jones et al., 1998; Nan et al., 1998; Jaenisch and Bird, 2003). Indeed, the present study demonstrated a significant decrease in the binding of Egr-1 to GR promoter 1₇ and Sp1 to GR promoter 1₁₁, respectively, providing a novel mechanism in fetal stress-induced down-regulation of GR expression in the brain at the molecular level. Among the transcription factor binding sites defined on GR promoters, the NGFI-A (Egr-1) binding site at rat promoter 1₇ and equivalent human promoter 1F has been extensively studied in perinatal programming of GR expression and subsequent disease (Weaver et al., 2004). Sp1 binding sites have also been reported to regulate GR gene expression (Suehiro et al., 2004). In agreement with the present study, previous studies demonstrated that fetal stress resulted in heightened CpG methylation and decreased Egr-1 and Sp1 binding to PKC epsilon promoter, leading to down-regulation of PKC epsilon expression in the developing heart (Patterson et al., 2010; Lawrence et al., 2011). It is thus plausible that transcription factors including Egr-1 and Sp1 and their corresponding binding sites on specific gene promoters are common targets of epigenetic regulation by perinatal environments.

The present investigation provides novel evidence that fetal hypoxia increases HI-induced brain injury in neonatal rats, providing new insights linking fetal stress and pathophysiological consequences of heightened HIE in newborns. Although it may be difficult to translate the present findings directly into humans, the possibility that fetal hypoxia may increase the risk of HIE in newborns provides a mechanistic understanding worthy of investigation in humans. This is because hypoxia is one of the most important and clinically relevant stresses to the fetus and because HIE occurs at a significantly

higher rate in compromised pregnancy and fetal development. The finding of fetal stress-mediated epigenetic repression of GR in the developing brain provides a novel mechanism in the understanding of HIE at the molecular level, and may suggest new insights for the development of new therapeutic strategies that may be beneficial for the treatment of newborns with HIE.

References

- Abraham, I. M., Harkany, T., Horvath, K. M., & Luiten, P. G. (2001). Action of glucocorticoids on survival of nerve cells: promoting neurodegeneration or neuroprotection? *Journal of neuroendocrinology*, *13*(9), 749-760.
- Barks, J. D., Post, M., & Tuor, U. I. (1991). Dexamethasone prevents hypoxic-ischemic brain damage in the neonatal rat. *Pediatric research*, *29*(6), 558-563. doi: 10.1203/00006450-199106010-00008
- Bertram, C., Trowern, A. R., Copin, N., Jackson, A. A., & Whorwood, C. B. (2001). The maternal diet during pregnancy programs altered expression of the glucocorticoid receptor and type 2 11beta-hydroxysteroid dehydrogenase: potential molecular mechanisms underlying the programming of hypertension in utero. *Endocrinology*, *142*(7), 2841-2853.
- Douglas-Escobar, M., & Weiss, M. D. (2012). Biomarkers of hypoxic-ischemic encephalopathy in newborns. *Frontiers in neurology*, *3*, 144. doi: 10.3389/fneur.2012.00144
- Feng, Y., Rhodes, P. G., & Bhatt, A. J. (2011). Dexamethasone pre-treatment protects brain against hypoxic-ischemic injury partially through up-regulation of vascular endothelial growth factor A in neonatal rats. *Neuroscience*, *179*, 223-232. doi: 10.1016/j.neuroscience.2011.01.050
- Flavin, M. P. (1996). Influence of dexamethasone on neurotoxicity caused by oxygen and glucose deprivation in vitro. *Experimental neurology*, *139*(1), 34-38. doi: 10.1006/exnr.1996.0078
- Graham, E. M., Ruis, K. A., Hartman, A. L., Northington, F. J., & Fox, H. E. (2008). A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. *American journal of obstetrics and gynecology*, *199*(6), 587-595. doi: 10.1016/j.ajog.2008.06.094
- Guy, J., Cheval, H., Selfridge, J., & Bird, A. (2011). The role of MeCP2 in the brain. *Annu Rev Cell Dev Biol*, *27*, 631-652. doi: 10.1146/annurev-cellbio-092910-154121
- Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature genetics*, *33 Suppl*, 245-254. doi: 10.1038/ng1089
- Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., . . . Wolffe, A. P. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nature genetics*, *19*(2), 187-191. doi: 10.1038/561

- Kauffman, K. S., Seidler, F. J., & Slotkin, T. A. (1994). Prenatal dexamethasone exposure causes loss of neonatal hypoxia tolerance: cellular mechanisms. *Pediatric research*, *35*(5), 515-522.
- Kurinczuk, J. J., White-Koning, M., & Badawi, N. (2010). Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. *Early human development*, *86*(6), 329-338. doi: 10.1016/j.earlhumdev.2010.05.010
- Lawrence, J., Chen, M., Xiong, F., Xiao, D., Zhang, H., Buchholz, J. N., & Zhang, L. (2011). Foetal nicotine exposure causes PKCepsilon gene repression by promoter methylation in rat hearts. *Cardiovascular research*, *89*(1), 89-97. doi: 10.1093/cvr/cvq270
- Li, Y., Gonzalez, P., & Zhang, L. (2012). Fetal stress and programming of hypoxic/ischemic-sensitive phenotype in the neonatal brain: mechanisms and possible interventions. *Progress in neurobiology*, *98*(2), 145-165. doi: 10.1016/j.pneurobio.2012.05.010
- Li, Y., Xiao, D., Dasgupta, C., Xiong, F., Tong, W., Yang, S., & Zhang, L. (2012). Perinatal nicotine exposure increases vulnerability of hypoxic-ischemic brain injury in neonatal rats: role of angiotensin II receptors. *Stroke; a journal of cerebral circulation*, *43*(9), 2483-2490. doi: 10.1161/STROKEAHA.112.664698
- Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., . . . Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science*, *277*(5332), 1659-1662.
- McCormick, J. A., Lyons, V., Jacobson, M. D., Noble, J., Diorio, J., Nyirenda, M., . . . Chapman, K. E. (2000a). 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. *Molecular endocrinology*, *14*(4), 506-517.
- McCormick, J. A., Lyons, V., Jacobson, M. D., Noble, J., Diorio, J., Nyirenda, M., . . . Chapman, K. E. (2000b). 5'-heterogeneity of glucocorticoid receptor messenger RNA is tissue specific: differential regulation of variant transcripts by early-life events. *Mol Endocrinol*, *14*(4), 506-517.
- Meyer, K., Zhang, H., & Zhang, L. (2009). Direct effect of cocaine on epigenetic regulation of PKCepsilon gene repression in the fetal rat heart. *Journal of molecular and cellular cardiology*, *47*(4), 504-511. doi: 10.1016/j.yjmcc.2009.06.004
- Mueller, B. R., & Bale, T. L. (2008). Sex-specific programming of offspring emotionality after stress early in pregnancy. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *28*(36), 9055-9065. doi: 10.1523/JNEUROSCI.1424-08.2008

Mulligan, C. J., D'Errico, N. C., Stees, J., & Hughes, D. A. (2012). Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. *Epigenetics*, 7(8), 853-857. doi: 21180 [pii]

10.4161/epi.21180

Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., & Bird, A. (1998). Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex. *Nature*, 393(6683), 386-389. doi: 10.1038/30764

O'Donnell, D., Larocque, S., Seckl, J. R., & Meaney, M. J. (1994). Postnatal handling alters glucocorticoid, but not mineralocorticoid messenger RNA expression in the hippocampus of adult rats. *Brain Res Mol Brain Res*, 26(1-2), 242-248.

Oberlander, T. F., Weinberg, J., Papsdorf, M., Grunau, R., Misri, S., & Devlin, A. M. (2008a). Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics*, 3(2), 97-106. doi: 6034 [pii]

Oberlander, T. F., Weinberg, J., Papsdorf, M., Grunau, R., Misri, S., & Devlin, A. M. (2008b). Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. *Epigenetics : official journal of the DNA Methylation Society*, 3(2), 97-106.

Patterson, A. J., Chen, M., Xue, Q., Xiao, D., & Zhang, L. (2010). Chronic prenatal hypoxia induces epigenetic programming of PKC{epsilon} gene repression in rat hearts. *Circulation research*, 107(3), 365-373. doi: 10.1161/CIRCRESAHA.110.221259

Rice, J. E., 3rd, Vannucci, R. C., & Brierley, J. B. (1981). The influence of immaturity on hypoxic-ischemic brain damage in the rat. *Annals of neurology*, 9(2), 131-141. doi: 10.1002/ana.410090206

Suderman, M., McGowan, P. O., Sasaki, A., Huang, T. C., Hallett, M. T., Meaney, M. J., . . . Szyf, M. (2012). Conserved epigenetic sensitivity to early life experience in the rat and human hippocampus. *Proc Natl Acad Sci U S A*, 109 Suppl 2, 17266-17272. doi: 1121260109 [pii]

10.1073/pnas.1121260109

Suehiro, T., Kaneda, T., Ikeda, Y., Arai, K., Kumon, Y., & Hashimoto, K. (2004). Regulation of human glucocorticoid receptor gene transcription by Sp1 and p53. *Mol Cell Endocrinol*, 222(1-2), 33-40. doi: 10.1016/j.mce.2004.05.005

S0303720704002126 [pii]

- Tomalski, P., & Johnson, M. H. (2010). The effects of early adversity on the adult and developing brain. *Current opinion in psychiatry*, 23(3), 233-238. doi: 10.1097/YCO.0b013e3283387a8c
- Tombaugh, G. C., Yang, S. H., Swanson, R. A., & Sapolsky, R. M. (1992). Glucocorticoids exacerbate hypoxic and hypoglycemic hippocampal injury in vitro: biochemical correlates and a role for astrocytes. *Journal of neurochemistry*, 59(1), 137-146.
- Towfighi, J., Yager, J. Y., Housman, C., & Vannucci, R. C. (1991). Neuropathology of remote hypoxic-ischemic damage in the immature rat. *Acta neuropathologica*, 81(5), 578-587.
- Tuor, U. I. (1995). Dexamethasone and the prevention of neonatal hypoxic-ischemic brain damage. *Annals of the New York Academy of Sciences*, 765, 179-195; discussion 196-177.
- Tuor, U. I. (1997). Glucocorticoids and the prevention of hypoxic-ischemic brain damage. *Neuroscience and biobehavioral reviews*, 21(2), 175-179.
- Tuor, U. I., Yager, J. Y., Bascaramurty, S., & Del Bigio, M. R. (1997). Dexamethasone prevents hypoxia/ischemia-induced reductions in cerebral glucose utilization and high-energy phosphate metabolites in immature brain. *Journal of neurochemistry*, 69(5), 1954-1963.
- Turner, J. D., Alt, S. R., Cao, L., Vernocchi, S., Trifonova, S., Battello, N., & Muller, C. P. (2010). Transcriptional control of the glucocorticoid receptor: CpG islands, epigenetics and more. *Biochemical pharmacology*, 80(12), 1860-1868. doi: 10.1016/j.bcp.2010.06.037
- Turner, J. D., & Muller, C. P. (2005). Structure of the glucocorticoid receptor (NR3C1) gene 5' untranslated region: identification, and tissue distribution of multiple new human exon 1. *Journal of molecular endocrinology*, 35(2), 283-292. doi: 10.1677/jme.1.01822
- Turner, J. D., Pelascini, L. P., Macedo, J. A., & Muller, C. P. (2008). Highly individual methylation patterns of alternative glucocorticoid receptor promoters suggest individualized epigenetic regulatory mechanisms. *Nucleic acids research*, 36(22), 7207-7218. doi: 10.1093/nar/gkn897
- Turner, J. D., Schote, A. B., Macedo, J. A., Pelascini, L. P., & Muller, C. P. (2006). Tissue specific glucocorticoid receptor expression, a role for alternative first exon usage? *Biochemical pharmacology*, 72(11), 1529-1537. doi: 10.1016/j.bcp.2006.07.005

- Vannucci, R. C., & Vannucci, S. J. (2005). Perinatal hypoxic-ischemic brain damage: evolution of an animal model. *Developmental neuroscience*, 27(2-4), 81-86. doi: 10.1159/000085978
- Weaver, I. C., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., . . . Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nat Neurosci*, 7(8), 847-854. doi: 10.1038/nn1276
- nn1276 [pii]
- Weaver, I. C., Champagne, F. A., Brown, S. E., Dymov, S., Sharma, S., Meaney, M. J., & Szyf, M. (2005). Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 25(47), 11045-11054. doi: 10.1523/JNEUROSCI.3652-05.2005
- Weaver, I. C., D'Alessio, A. C., Brown, S. E., Hellstrom, I. C., Dymov, S., Sharma, S., . . . Meaney, M. J. (2007). The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 27(7), 1756-1768. doi: 10.1523/JNEUROSCI.4164-06.2007
- Weaver, I. C., Diorio, J., Seckl, J. R., Szyf, M., & Meaney, M. J. (2004). Early environmental regulation of hippocampal glucocorticoid receptor gene expression: characterization of intracellular mediators and potential genomic target sites. *Annals of the New York Academy of Sciences*, 1024, 182-212. doi: 10.1196/annals.1321.099
- Whitelaw, A., & Thoresen, M. (2000). Antenatal steroids and the developing brain. *Archives of disease in childhood. Fetal and neonatal edition*, 83(2), F154-157.
- Xiong, F., Xiao, D., & Zhang, L. (2012). Norepinephrine causes epigenetic repression of PKCepsilon gene in rodent hearts by activating Nox1-dependent reactive oxygen species production. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 26(7), 2753-2763. doi: 10.1096/fj.11-199422
- Xiong, F., & Zhang, L. Role of hypothalamic-pituitary-adrenal axis in developmental programming of health and disease. *Frontiers in neuroendocrinology*, Accepted with minor revision.
- Xue, Q., Dasgupta, C., Chen, M., & Zhang, L. (2011a). Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. *Cardiovascular research*, 89(2), 300-308. doi: 10.1093/cvr/cvq303

Xue, Q., Dasgupta, C., Chen, M., & Zhang, L. (2011b). Foetal hypoxia increases cardiac AT(2)R expression and subsequent vulnerability to adult ischaemic injury. *Cardiovasc Res*, 89(2), 300-308. doi: cvq303 [pii]

CHAPTER FOUR
DEXAMETHASONE PROTECTS NEONATAL HYPOXIC-
ISCHEMIC BRAIN INJURY VIA L-PGDS-DEPENDENT PGD₂-
DP₁-PERK SIGNALING PATHWAY

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Abstract

Dexamethasone treatment on neonate rat brain has demonstrated neuroprotection against hypoxic-ischemic brain injury. However, very little is known about this neuroprotective mechanism. We examined the effect of dexamethasone on the level of pERK 1/2 and determined the effect of inhibition of LPGDS, DP1 receptor and MAPK/ERK on the expression of pERK 1/2 in the presence and absence of dexamethasone. In total, 116 ten-day old rats were used. Dexamethasone and inhibitors were administered through intracerebroventricular injections. Effect on the level of pERK 1/2 expression patterns were assessed through western blotting. Prostaglandin D2 levels were determined by ELISA. HI brain injury volume was determined 48 hours after drug treatment. Dexamethasone increases the level of pERK-44 in normoxic brains effect that was repressed by the presence of PD-98059, an inhibitor of MAPK. Dexamethasone also increases the expression of LPGDS and the levels of PGD₂ in the P10 rat brains. Inhibitors of LPGDS and DP1 receptor also repressed the effect of dexamethasone in the expression of pERK-44. Moreover, the neuroprotective effect of dexamethasone against HI-brain injury was blocked by the presence of those inhibitors. Dexamethasone up-regulated the level of pERK-44 and the inhibition of the MAPK repressed the neuroprotective effect of dexamethasone suggesting that the neuroprotective mechanism of dexamethasone against HI brain injury is associated with the MAPK/ERK pathway, where LPGDS/PGD cascade plays a significant role.

Introduction

Glucocorticoids are essential for life and play a crucial role in the regulation of growth and development. The brain is the major neural target for the action of GCs, where they bind to intracellular glucocorticoid and mineralocorticoid receptors in neurons and glia (Abraham, Harkany, Horvath, & Luiten, 2001). GRs are highly expressed in the developing brain and a neuroprotective role against hypoxia-ischemic injury has been reported for dexamethasone a synthetic glucocorticoid (Gonzalez-Rodriguez, Xiong, Li, Zhou, & Zhang, 2014). The neuroprotective effects of GCs may be mediated by distinct mechanisms involving modulation of Ca^{2+} currents, enhanced synthesis of lipocortin-1 and neurotrophic factors, and their ability to attenuate lipid peroxidation (Flower & Rothwell, 1994; Joels & de Kloet, 1994; Mocchetti, Spiga, Hayes, Isackson, & Colangelo, 1996; Young & Flamm, 1982). However, the mechanism of neuroprotection for dexamethasone against hypoxia ischemia in neonate rats is not well understood. The intracellular mitogen activated protein kinase/extracellular signal regulated kinase signaling pathway is used by neurotrophins, neurotransmitters, and neuropeptides to exert their neurotrophic and neuroprotective effects by specifically enhancing progenitor cell proliferation and differentiation, neuronal process growth and regeneration, neuronal survival, and long-term synaptic remodeling and plasticity (Chen & Manji, 2006; Huang, Wu, Chen, Manji, & Chen, 2003; Sweatt, 2004).

Prostaglandin D synthase catalyzes the isomerization of PGH_2 , synthesized by cyclooxygenase (COX)-2, to produce PGD_2 (Smith, Marnett, & DeWitt, 1991; Urade & Hayaishi, 2000). Two types of PGDS have been purified and characterized; lipocalin-type PGDS that was previously known as the brain-type enzyme or glutathione-independent enzyme, and the hematopoietic PGDS, also known as the spleen-type

enzyme or GSH-requiring enzymes (Kanaoka & Urade, 2003; A. Nagata et al., 1991). Lipocalin-type PGDS is a unique bi-functional protein, is the only member of the lipocalin family with and associated enzymatic activity: the synthesis of prostaglandin D₂ in the central nervous system, cardiovascular system and in ocular tissues among others (Beuckmann et al., 1996; Beuckmann et al., 2000; Eguchi et al., 1997). Moreover, L-PGDS is an extracellular transporter for lipophilic ligands such as retinoids and tyroids, retinoic acid and amyloid β peptides (Kanekiyo et al., 2007; Pervaiz & Brew, 1987; Tanaka et al., 1997). Recently, it has been reported that LPGDS protected against neuronal cell death due to oxidative stress and HIE (Fukuhara et al., 2012; Taniguchi, Mohri, Okabe-Arahoru, Kanekiyo, et al., 2007). Prostaglandin D₂ an arachidonic acid metabolite, is the most abundant prostaglandin in the brain, (Hertting & Seregi, 1989) where it regulates sleep, temperature and nociception through two distinct G protein-coupled receptors, DP/DP1 receptor and DP2/CRTH2 (Kabashima & Narumiya, 2003; K. Nagata & Hirai, 2003). Although PGD₂ has been associated with inflammation in peripheral tissues (Honda et al., 2003), and inhibition of platelet aggregation (Yun, Ohman, Gill, & Keiser, 1991), *in-vitro* and *in-vivo* studies have demonstrated a neuroprotective role for PGD₂ (Liang, Wu, Hand, & Andreasson, 2005; Taniguchi, Mohri, Okabe-Arahoru, Aritake, et al., 2007). In the present study, we examined if the arachidonic cascade and the MAPK/ERK pathway are part of the neuroprotective mechanism of glucocorticoids.

Material and Methods

Experimental Animals

Female Sprague Dawley rats with 10-day-old neonates were purchased from Charles River Laboratories (Portage, MI). All rats were kept in a room maintained at 24°C, a 12-h light/dark cycle, and provided *ad libitum* access to normal rat chow and filtered water. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

To evaluate the neuroprotective mechanism of dexamethasone, inhibitors of LPGDS (SeCl₄, Sigma), MEK1/2 (PD98059, Sigma) and the antagonist of DP1 receptor (MK-0524, Santa Cruz) were used in presence and absence of dexamethasone. These were injected into the right lateral ventricle prior to the HI treatment. Pups were anesthetized and fixed on a stereotaxic apparatus (Stoeling). An incision was made on the skull surface and bregma exposed. Each reagent or a cocktail was injected at a rate of 1ul/minute with a 10 ul syringe (Stoeling) on the right hemisphere following the coordinates relative to bregma: 2 mm posterior, 1.5 mm lateral and 3.0 mm below the skull surface (Li, Gonzalez, & Zhang, 2012). Saline was injected as the vehicle control. The injection lasted 2 minutes and the needle was kept for additional 5 minutes before its removal. The incision was sutured, and the animals were allowed to recover on a 37°C heated blanket. The animals were returned to their dams after recovering from anesthesia. Dexamethasone and SeCl₄ were purchased from Sigma.

Prostaglandin Quantification

Forty-eight hours after dexamethasone was injected to the P-10 pups,

dexamethasone and control group were decapitated, the amount of PGs in their fresh-frozen right hemisphere brain was determined by ELISA (Arnold, 2012), following the manufacturer's instruction with slight modification. This kit is based on the conversion of PGD₂ to a stable methoxime derivative by treatment with methoxamine hydrochloride. Briefly, the right hemispheres were homogenized with 50mM KH₂PO₄, 5mM EDTA, 43 mM acetylsalicylic acid and pH 7.4 buffer. Homogenate were centrifuged at 13,600 g for 5 min and supernatants stored at -80 ° C. Samples were diluted with cold acetone, incubated on ice for 5 min and centrifuged at 3,000 rpm for 10 min. Frozen sample were lyophilized. PGD₂ concentration was measured using a PGD-Methoxime EIA kit (Cayman Chemicals).

Western Blot

Brains were homogenized on an ice-cold lysis buffer containing 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 50 mM Tris-HCl, 10 mM EDTA, 0.1% Tween-20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 ug/ml leupatin and 5 ug/ml aprotinin, pH 7.4. Homogenates were centrifuged at 4°C for 10 min at 14,000g, and supernatants aliquots were collected and stored at -80°C. Protein concentrations were determined using a protein assay kit from Bio-Rad. Samples with equal protein (40 μg) were loaded on 10% sodium dodecyl sulfate polyacrylamide gel and performed the electrophoresis analysis. After electrophoresis, proteins were transferred to nitrocellulose membranes. Nonspecific binding was blocked in TBST containing 5% dry milk for 60 min at room temperature. The membranes were incubated with rabbit LPGDS, DP1 (1:200; Santa Cruz Biotechnology) and rabbit pERK1/2 (1:1000; Cell Signaling)

polyclonal antibody overnight at 4°C. The membranes were then washed and incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized with enhanced chemiluminescence reagents, and the blots were exposed to Hyperfilm (GE Healthcare). Results were analyzed and quantified by the Kodak electrophoresis documentation and analysis system with Kodak ID image analysis software. For comparison of the levels of LPGDS, DP1 and pERK1/2 protein relative density between the groups, samples were normalized first to β -actin values and then presented as fold values relative to sham-treated animals.

Brain Hypoxic-Ischemic Treatment

Functional studies were performed by inducing brain HI injury in P10 rat pups, using a modified Rice-Vannucci model, described previously (Gonzalez-Rodriguez et al., 2014). Briefly, pups were anesthetized with 2% isoflurane, a small incision was made in the right side of the neck where the right common carotid artery was exposed and ligated with silk surgical suture. The incision was sutured. After recovery for 1 hour, pups were treated with 8% O₂ for 2 hours. Following 3 hours of recovery, on a warm pad, the pups were returned to their moms.

Brain Infarction Measurement

Forty-eight hours after HI treatment pups were euthanized and brain infarct size was determined as previously described (Gonzalez-Rodriguez et al., 2014). Briefly, brain coronal slices of 2-mm thick were immersed in a 2% solution of 2,3,5-

triphenyltetrazolium chloride monohydrate (Sigma-Aldrich) for 5 min at 37 °C and then fixed by 10% formaldehyde overnight. Each slice was weighed, photographed separately and the percentage of infarction area for each slice was analyzed by Image J software (Version 1.40; National Institute of Health, Bethesda, MD), corrected by slice weight, summed for each brain, and expressed as a percentage of whole brain weight.

Statistical Analysis

Data are expressed as mean \pm SEM. Experimental number (n) represents neonates from different dams. Data was analyzed by Student's t-test using GraphPad Prism (v5.0a). Values of $P < 0.05$ were considered as significant.

Results

Brain Protein Levels of pERK 1/2 Affected by Dexamethasone Treatment

To analyze the cell signaling pathway responsible for dexamethasone neuroprotection, we prepared Western blots with tissue samples from P10 rat brain. Dexamethasone treatment on P10 rat showed a significant increase of the expression of pERK-44 (* $p < 0.05$ versus saline; Figure 1) on the brain, while there was no effect of the dexamethasone treatment on the expression of isoform pERK-42 (* $p > 0.05$ versus saline; Figure 1A). To demonstrate that the effect on the expression of pERK-44 was associated with dexamethasone treatment, inhibition of MEKK with PD98059 was evaluated. P10 rats treated with PD98059 showed no effect on the protein expression of pERK-44 after dexamethasone treatment (* $p > 0.05$ versus PD98059; Figure 1B).

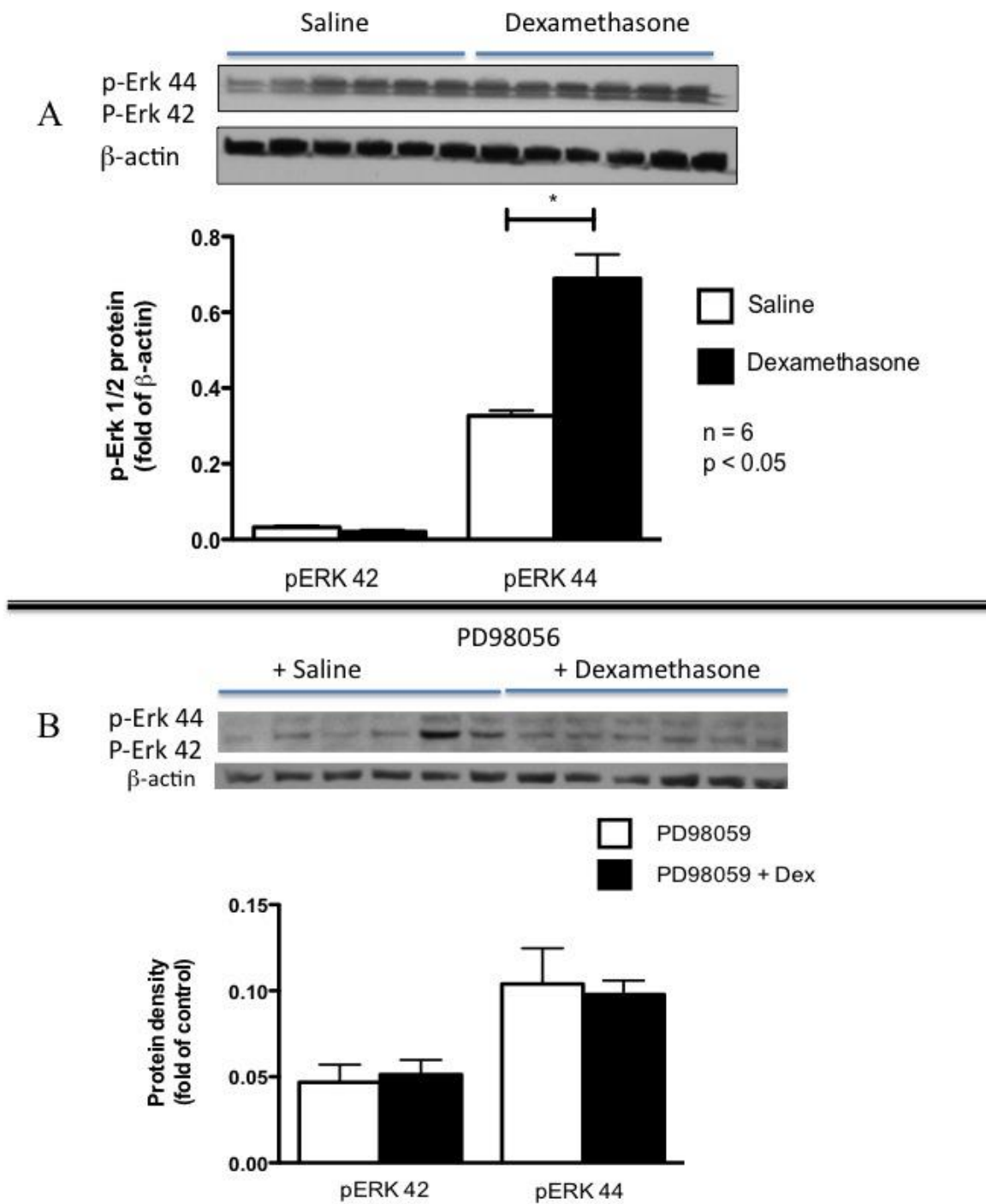


Figure 1. Dexamethasone effect on the protein abundance of phosphorylated extracellular signal-regulated kinases and the blocking effect on dexamethasone by MAPK/ERK inhibitor, PD98059. A Dexamethasone increase the protein expression of pERK-44 (* $p < 0.05$ vs control; $n = 6$ each group) but has no effect on pERK-42 ($n = 6$ each group). B No effects of dexamethasone on the protein density level of pERK 1/2 were observed in the presences of PD98059 ($n = 6$ each group). Values are expressed as a mean \pm SEM.

Prostaglandin D2 Level was Increased by Dexamethasone

We quantified the level of prostaglandin D2 in the brain of P10 rats 48 h after dexamethasone treatment. The level of PGD2 was significantly increased (* $p < 0.05$ versus control; Figure 2) by the presence of the glucocorticoid. The level of LPGDS was also determined in the presence of dexamethasone. As shown in Fig. 3 the protein expression of LPGDS was significantly increased (* $p < 0.05$ versus control) 48 h after dexamethasone treatment. Figure 4 shows western blot analysis of DP1 receptor on neonate brains after the dexamethasone treatment. No significant difference was observed ($p > 0.05$ versus control).

Inhibition of LPGDS and the Effect of Dexamethasone on pERK 1/2

We examined the effect of dexamethasone on P10 brain rats treated with SeCl₄, a selective inhibitor of LPGDS, in the presence and absence of dexamethasone. The quantitative analysis of the Western blot is shown in Figure 5A where the expression of pERK 1/2 is not affected by the presence of dexamethasone ($p > 0.05$ versus SeCl₄) when LPGDS is inhibited by selenium chloride.

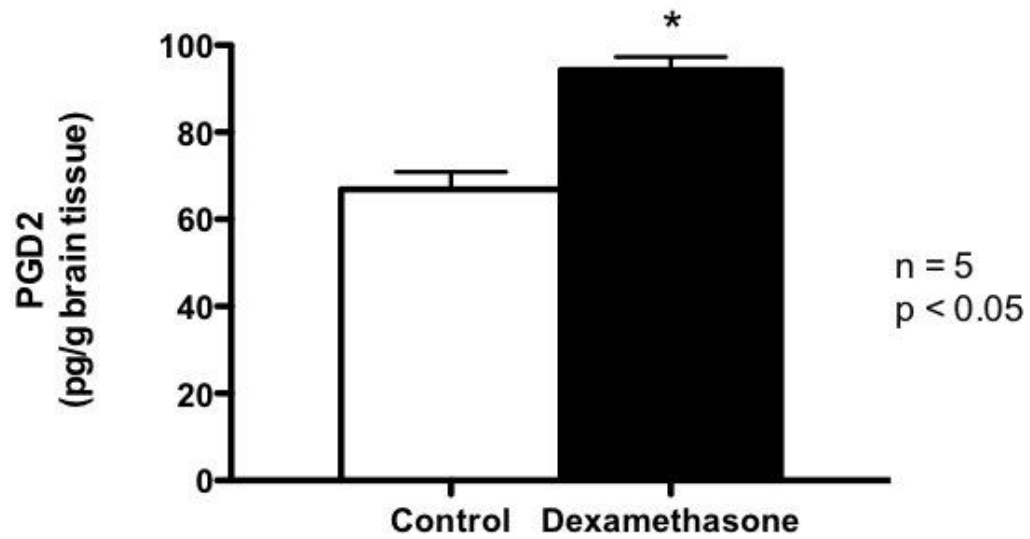


Figure 2. Prostaglandin D2 levels in the brain of neonate rat brains after intracerebroventricular injection of dexamethasone or saline. Dexamethasone increases the levels of PGD₂ in the normoxic brains (*p<0.05 vs control). PGD₂ from 6 rats, for each treatment, was obtained and analyzed by ELISA. Data are expressed as a mean±SEM.

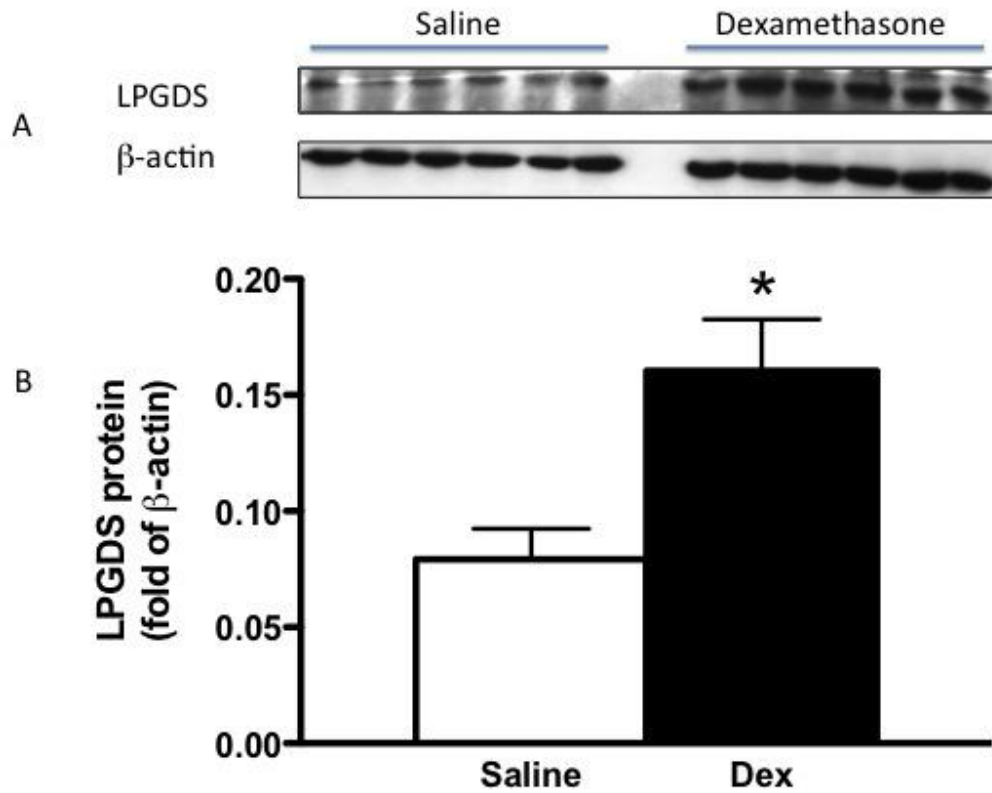


Figure 3. Effect of dexamethasone on the protein expression of lipocalin-type prostaglandin D synthase. A Representative Western blots of LPGDS and b-actin for brain samples treated with dexamethasone or saline. B Quantitative analysis of the protein expression for LPGDS. The dexamethasone treatment increases the protein level of LPGDS (* $p < 0.05$ vs control, $n = 6$). Data are expressed as a mean \pm SEM.

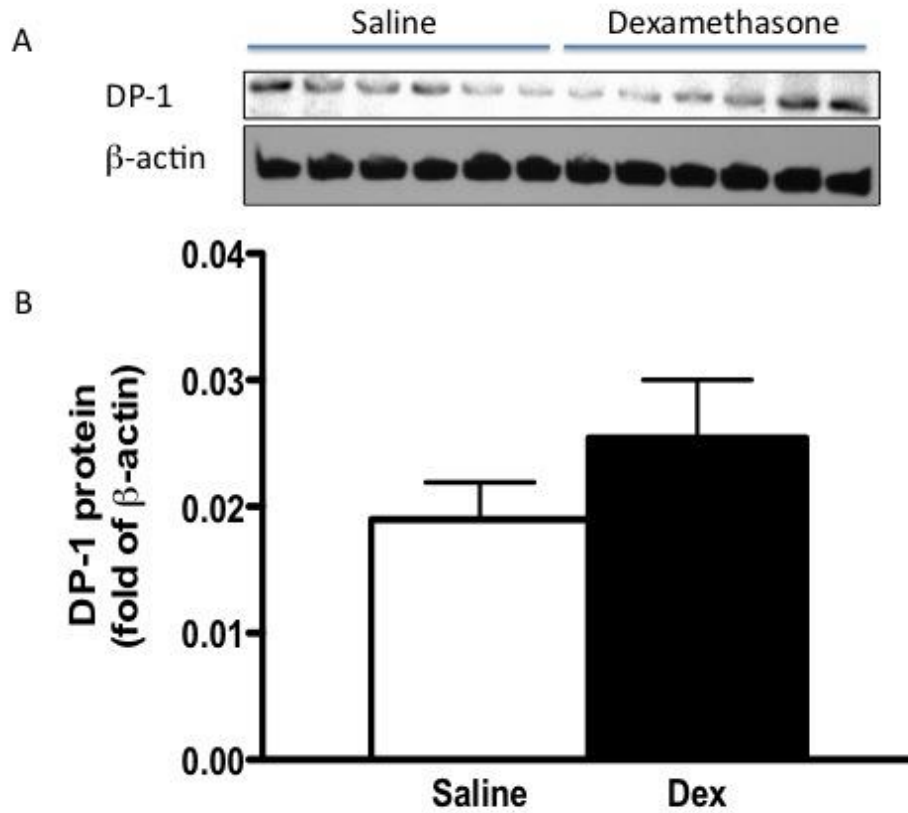


Figure 4. Expression of prostaglandin D2 receptor in the brain of neonate rat after dexamethasone treatment. A Representative Western blots of DP1 receptor for brain samples treated with dexamethasone or saline, normalized to b-actin. B Quantitative analysis of the protein expression for DP1. No effect was found on the protein levels of DP1, 48 hours after dexamethasone treatment (n=6). Data are expressed as a mean±SEM.

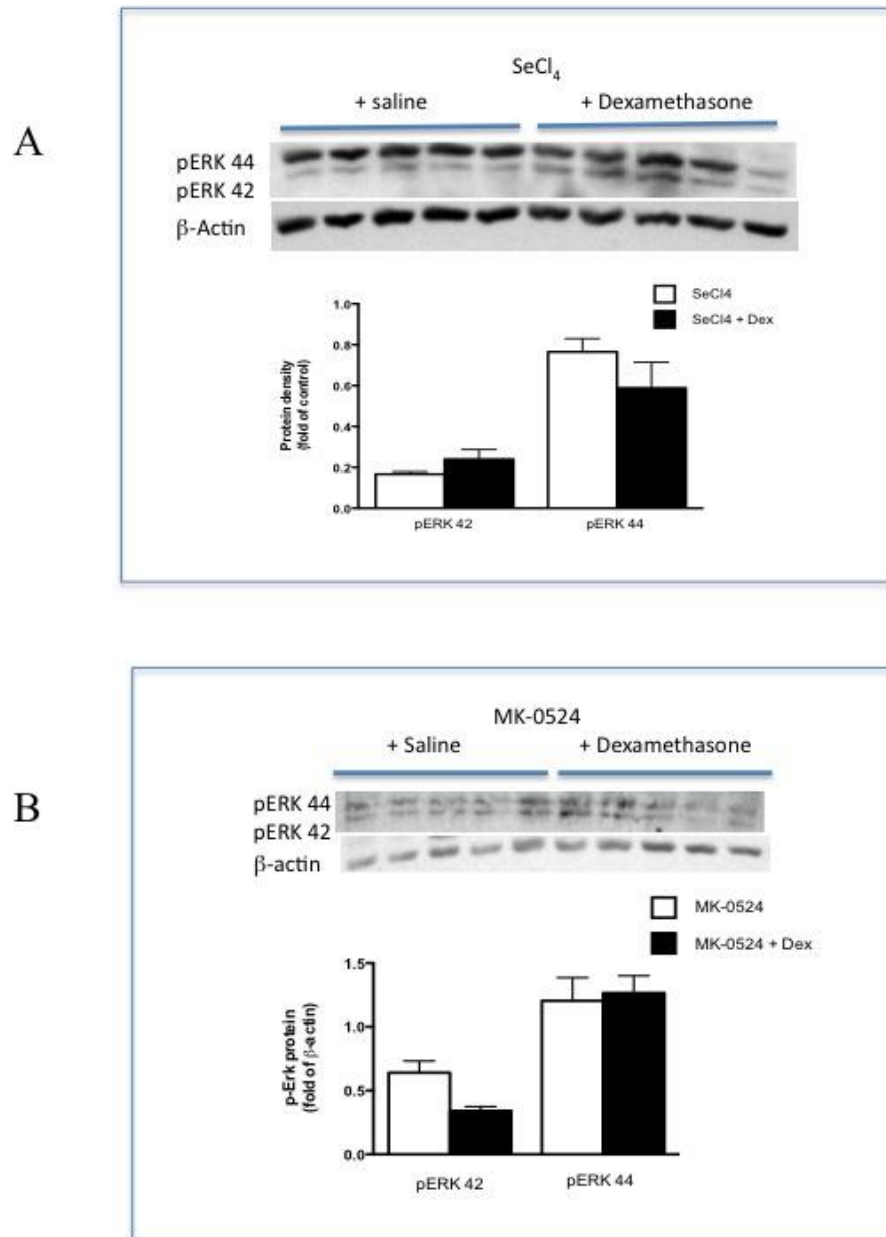


Figure 5. Representative Western blots and quantitative analysis of phosphorylated extracellular signal-regulated kinases. A Effect of dexamethasone in the protein expression of pERK on rat brain treated with selenium chloride, a selective inhibitor of lipocalin-type prostaglandin D synthase. The dexamethasone has no effect on the protein level of pERK in the presence of the SeCl₄ (n=5). B Dexamethasone effect in the protein levels of pERK on rat brain treated with MK-0524, a selective antagonist for prostaglandin D2 receptor. The dexamethasone has no effect on the protein level of pERK in the presence of the MK-0524 (n=5). Data are expressed as a mean±SEM.

Blocking of DP1 Receptors and the Effect of Dexamethasone on Protein Level of pERK^{1/2}

The possible role of prostaglandin D2 in the neuroprotective mechanism of dexamethasone was evaluated by treatment of MK-0524, a DP1-selective antagonist, in the presence and absence of dexamethasone in P10 rat brain. Figure 5B shows that the protein levels of pERK 1/2 were not affected by the presence of dexamethasone ($p > 0.05$ versus MK-0524).

Dexamethasone's Neuroprotective Role In Vivo is Associated with pERK Pathway

The HI brain injury model was used to evaluate the neuroprotective pathway associated with dexamethasone treatment. We applied signal transduction inhibitor PD98059 into intracerebroventricular space in the presence and absence of dexamethasone. Figure 6A shows the neuroprotective effect of dexamethasone on the brain exposed to HI brain injury, 4.56 ± 0.65 (Figure 6Aii) versus 10.95 ± 1.46 (Figure 6Ai) % infarction ($*p < 0.05$), this neuroprotective effect was repressed by the presence of the MEKK inhibitor, 16.77 ± 3.00 (Figure 6Aiv) versus 18.47 ± 3.03 (Figure 6 Aiii) % infarction. Treatment of the inhibitor of LPGDS in the neonate brains reversed the neuroprotective effect of dexamethasone, 16.38 ± 1.70 (Figure 6Avi) versus 15.10 ± 2.25 (Figure 6Av) % infarction. Similar results were observed when the brains were treated with the DP1 antagonist, MK-0524 where dexamethasone could not protect against the HI brain injury, in the presence of the antagonist (18.36 ± 1.34 versus 18.32 ± 0.42 % infarction, Figure 6Avii and Figure 6Aviii).

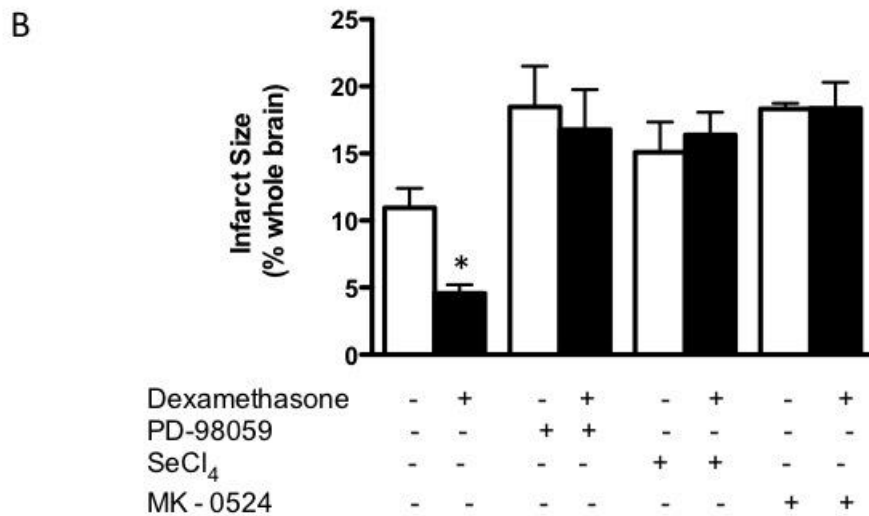
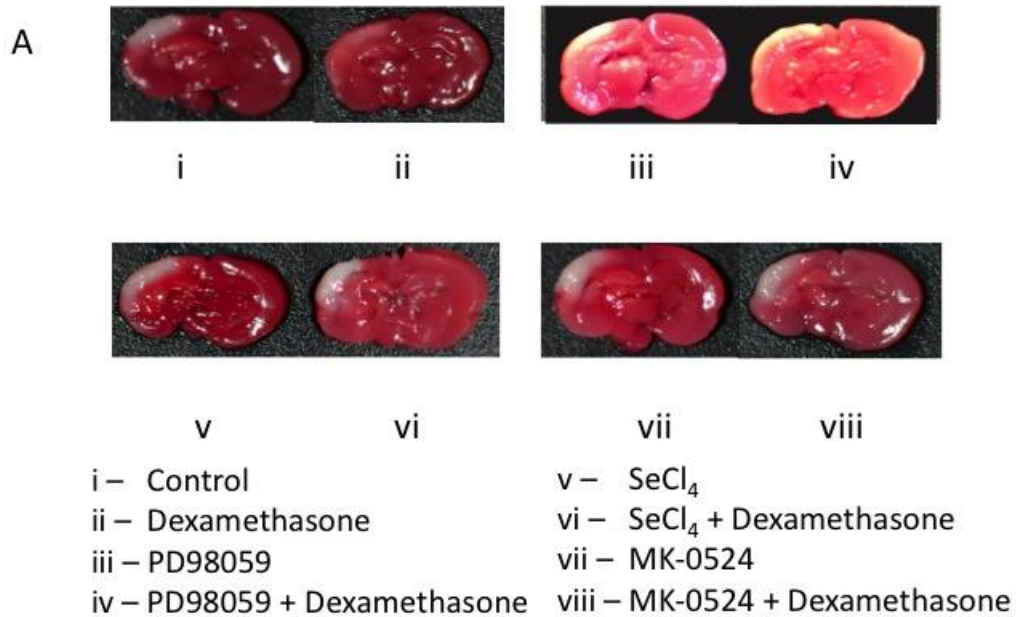


Figure 6. Neuroprotective effect of dexamethasone against HI brain injury in P10 pups. A Brain infarct volume was determined at 48 hours after permanent MCAO by TTC. A total of eight groups were evaluated (n=6 for each group). B Quantitative analysis of the brain infarction size. The group treated only with dexamethasone has the lower infarction volume (*p<0.05 vs control). This neuroprotection was block in the presence of the inhibitor for MAPK/ERK inhibitor, PD98059. Similar results were observed for the groups treated with the inhibitor of LPGDS, SeCl₄, and with the antagonist of DP1 receptor, MK-0524 (n=6 for each group). Data are expressed as a mean±SEM.

Discussion

The new findings of the present study are: (1) dexamethasone increases selectively the expression of the isoform of pERK-44 on P-10 neonate brains, (2) the interaction of arachidonic acid cascade in the neuroprotective mechanism of dexamethasone and (3) the neuroprotective role of dexamethasone against HI brain injury is associated with the MAPK/ERK pathway.

Previous studies have demonstrated that maternal hypoxia increase the vulnerability of the brain to HI injury by repressing the expression of glucocorticoid receptor. Also, that activation of GRs by dexamethasone reduced the HI-induced brain injury suggesting a neuroprotective role to GRs (Gonzalez-Rodriguez et al., 2014). However, the neuroprotective mechanism associated with GRs is not well understood. In this study we reported that dexamethasone treatment increased the protein expression of pERK-44, although the same treatment did not affect the expression of pERK-42, suggesting a selective regulation of pERK-44 by dexamethasone. In vitro studies, where morphine down-regulated selectively pERK-42 levels with no effect on pERK-44, have also demonstrated a selective regulation on pERK1/2 (Muller & Unterwald, 2004). Neonate brains treated with PD98059 repressed the effect of the up-regulation by dexamethasone, suggesting that pERK-44 could be the isoform of pERK that could regulate the neuroprotective mechanism of dexamethasone. The downstream part of the mechanism by which dexamethasone exerts this protective effect is associated with the blocking of the cascade of events culminating in apoptosis (Ekert et al., 1997). One pathway could include the phosphorylation of Thr125 in caspase-9, a conserved MAPK consensus site targeted by ERK2 *in vitro*, block the caspase-9 processing and subsequent caspase-3 activation (Allan et al., 2003).

Lipocalin-type prostaglandin D synthase catalyzes the isomerization of PGH₂, a common precursor of various prostanoids, to produce PGD₂, a potent endogenous somnogen and nociceptive modulator. This enzyme is the most abundant cerebrospinal fluid protein produced in the brain (Kanekiyo et al., 2007). LPGDS-deficient mice exhibited an exacerbated phenotype following transient or permanent ischemic brain injury, indicating a critical role of L-PGDS in protection against cerebral ischemia (Saleem, Shah, Urade, & Dore, 2009). HIE neonatal brains from human and mouse, expressed a higher number of L-PGDS-positive cells than in non-HIE neonatal brains, which were immunoreactive for p53, while some of these expressed cleaved caspase-3 (Taniguchi, Mohri, Okabe-Araori, Kanekiyo, et al., 2007). It has been demonstrated the neuroprotective role of PGD₂ *in vitro* (Liang et al., 2005), therefore, it is possible that LPGDS may protect the neurons by local secretion of PGD₂ in HIE brain. Also, dexamethasone treatment, in mouse neuronal cells, induces LPGDS mRNA and protein expression (Garcia-Fernandez et al., 2000). In our results dexamethasone treatment increased the protein levels of LPGDS in neonatal normoxic brains. To evaluate the possible role of LPGDS in the neuroprotective mechanism of dexamethasone neonate brains were treated with selenium chloride, and inhibitor for LPGDS, in the presence and absence of dexamethasone. Our data demonstrated that the effect of dexamethasone on the expression of pERK 44 was blocked by the presence of the LPGDS inhibitor and also that the neuroprotective effect of dexamethasone on HI brain injury was repressed by SeCl₄. This data suggest that LPGDS plays a significant role on the neuroprotective mechanism of dexamethasone toward HI brain injury that includes the activation of the MAPK/ERK pathway.

Prostaglandins are up regulated in the ischemic brains (Nogawa, Zhang, Ross, & Iadecola, 1997) and it has been reported that in mouse PGD₂ protects the brain from hypoxic ischemic injury (Taniguchi, Mohri, Okabe-Arahoru, Aritake, et al., 2007). Also an *in-vitro* study demonstrated that prostaglandin D2 activated the ERK mitogen-activated protein kinase (Choi et al., 2011). The results from our ELISA study showed that the levels of prostaglandin D2 increased due to the presence of glucocorticoids. Moreover, in our functional studies, brain treated with the DP1 antagonist, MK-0524, demonstrated that the neuroprotective effect of dexamethasone was blocked and at molecular levels dexamethasone has none effect in the expression of pERK 1/2. Our results suggested that dexamethasone increased the level of prostaglandin D2 that eventually induced neuroprotection through the DP1 receptor by activating the downstream kinase ERK 1/2 which is involved in the PGD₂-DP-mediated neuroprotection. Interestingly, it has been reported that PGD₂ exerted cardioprotective effect by binding to the DP receptor being ERK 1/2, the major downstream pathway, due to the presence of glucocorticoids (Tokudome et al., 2009).

Perinatal HI is a major cause of death and disability worldwide which has been limited to supportive intensive care. Although there are advances in research of cellular processes and molecular mechanism, hypothermia is the only treatment effective in neonatal HIE at present. The combination of hypothermia with neuroprotective treatments looks to be promising in a way to improve the neurological outcome of HI. In this study we demonstrated that the neuroprotective effect of dexamethasone on neonate HI brain injury is associated with the pathway of MAPK/ERK and that LPGDS/PGD cascade plays a significant role in this glucocorticoid neuroprotective mechanism. Future

studies related to the selective regulation of dexamethasone on the production of pERK44 needs to be done and the possibility that pERK44 could play a neuroprotective role needs to be elucidated. The downstream neuroprotective mechanism of glucocorticoids, that are associated with apoptosis, seems like a very fertile area that could be explored to find a novel treatment against neonates HI brain injury.

References

- Abraham, I. M., Harkany, T., Horvath, K. M., & Luiten, P. G. (2001). Action of glucocorticoids on survival of nerve cells: promoting neurodegeneration or neuroprotection? *J Neuroendocrinol*, *13*(9), 749-760.
- Allan, L. A., Morrice, N., Brady, S., Magee, G., Pathak, S., & Clarke, P. R. (2003). Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. *Nat Cell Biol*, *5*(7), 647-654. doi: 10.1038/ncb1005
- Arnold, W., Kim, P.Y., Allen, K.G.D. and Florant, G.L. (2012). Seasonal Variation in Brain Prostaglandin D2 and E2 of Marmots and n-6 Fatty Acid Availability *Living in a Seasonal World* (pp. 531-542). Berlin Heidelberg: Springer-Verlag.
- Beuckmann, C. T., Gordon, W. C., Kanaoka, Y., Eguchi, N., Marcheselli, V. L., Gerashchenko, D. Y., . . . Bazan, N. G. (1996). Lipocalin-type prostaglandin D synthase (beta-trace) is located in pigment epithelial cells of rat retina and accumulates within interphotoreceptor matrix. *J Neurosci*, *16*(19), 6119-6124.
- Beuckmann, C. T., Lazarus, M., Gerashchenko, D., Mizoguchi, A., Nomura, S., Mohri, I., . . . Urade, Y. (2000). Cellular localization of lipocalin-type prostaglandin D synthase (beta-trace) in the central nervous system of the adult rat. *J Comp Neurol*, *428*(1), 62-78.
- Chen, G., & Manji, H. K. (2006). The extracellular signal-regulated kinase pathway: an emerging promising target for mood stabilizers. *Curr Opin Psychiatry*, *19*(3), 313-323. doi: 10.1097/01.yco.0000218604.63463.cd
- Choi, Y. H., Lee, S. N., Aoyagi, H., Yamasaki, Y., Yoo, J. Y., Park, B., . . . Yoon, J. H. (2011). The extracellular signal-regulated kinase mitogen-activated protein kinase/ribosomal S6 protein kinase 1 cascade phosphorylates cAMP response element-binding protein to induce MUC5B gene expression via D-prostanoid receptor signaling. *J Biol Chem*, *286*(39), 34199-34214. doi: 10.1074/jbc.M111.247684
- Eguchi, Y., Eguchi, N., Oda, H., Seiki, K., Kijima, Y., Matsu-ura, Y., . . . Hayaishi, O. (1997). Expression of lipocalin-type prostaglandin D synthase (beta-trace) in human heart and its accumulation in the coronary circulation of angina patients. *Proc Natl Acad Sci U S A*, *94*(26), 14689-14694.
- Ekert, P., MacLusky, N., Luo, X. P., Lehotay, D. C., Smith, B., Post, M., & Tanswell, A. K. (1997). Dexamethasone prevents apoptosis in a neonatal rat model of hypoxic-ischemic encephalopathy (HIE) by a reactive oxygen species-independent mechanism. *Brain Res*, *747*(1), 9-17.
- Flower, R. J., & Rothwell, N. J. (1994). Lipocortin-1: cellular mechanisms and clinical relevance. *Trends Pharmacol Sci*, *15*(3), 71-76.

- Fukuhara, A., Yamada, M., Fujimori, K., Miyamoto, Y., Kusumoto, T., Nakajima, H., & Inui, T. (2012). Lipocalin-type prostaglandin D synthase protects against oxidative stress-induced neuronal cell death. *Biochem J*, *443*(1), 75-84. doi: 10.1042/BJ20111889
- Garcia-Fernandez, L. F., Iniguez, M. A., Eguchi, N., Fresno, M., Urade, Y., & Munoz, A. (2000). Dexamethasone induces lipocalin-type prostaglandin D synthase gene expression in mouse neuronal cells. *J Neurochem*, *75*(2), 460-470.
- Gonzalez-Rodriguez, P. J., Xiong, F., Li, Y., Zhou, J., & Zhang, L. (2014). Fetal hypoxia increases vulnerability of hypoxic-ischemic brain injury in neonatal rats: Role of glucocorticoid receptors. *Neurobiol Dis*, *65C*, 172-179. doi: 10.1016/j.nbd.2014.01.020
- Hertting, G., & Seregi, A. (1989). Formation and function of eicosanoids in the central nervous system. *Ann N Y Acad Sci*, *559*, 84-99.
- Honda, K., Arima, M., Cheng, G., Taki, S., Hirata, H., Eda, F., . . . Fukuda, T. (2003). Prostaglandin D2 reinforces Th2 type inflammatory responses of airways to low-dose antigen through bronchial expression of macrophage-derived chemokine. *J Exp Med*, *198*(4), 533-543. doi: 10.1084/jem.20022218
- Huang, X., Wu, D. Y., Chen, G., Manji, H., & Chen, D. F. (2003). Support of retinal ganglion cell survival and axon regeneration by lithium through a Bcl-2-dependent mechanism. *Invest Ophthalmol Vis Sci*, *44*(1), 347-354.
- Joels, M., & de Kloet, E. R. (1994). Mineralocorticoid and glucocorticoid receptors in the brain. Implications for ion permeability and transmitter systems. *Prog Neurobiol*, *43*(1), 1-36.
- Kabashima, K., & Narumiya, S. (2003). The DP receptor, allergic inflammation and asthma. *Prostaglandins Leukot Essent Fatty Acids*, *69*(2-3), 187-194.
- Kanaoka, Y., & Urade, Y. (2003). Hematopoietic prostaglandin D synthase. *Prostaglandins Leukot Essent Fatty Acids*, *69*(2-3), 163-167.
- Kanekiyo, T., Ban, T., Aritake, K., Huang, Z. L., Qu, W. M., Okazaki, I., . . . Urade, Y. (2007). Lipocalin-type prostaglandin D synthase/beta-trace is a major amyloid beta-chaperone in human cerebrospinal fluid. *Proc Natl Acad Sci U S A*, *104*(15), 6412-6417. doi: 10.1073/pnas.0701585104
- Li, Y., Gonzalez, P., & Zhang, L. (2012). Fetal stress and programming of hypoxic/ischemic-sensitive phenotype in the neonatal brain: mechanisms and possible interventions. *Prog Neurobiol*, *98*(2), 145-165. doi: 10.1016/j.pneurobio.2012.05.010

- Liang, X., Wu, L., Hand, T., & Andreasson, K. (2005). Prostaglandin D2 mediates neuronal protection via the DP1 receptor. *J Neurochem*, *92*(3), 477-486. doi: 10.1111/j.1471-4159.2004.02870.x
- Mocchetti, I., Spiga, G., Hayes, V. Y., Isackson, P. J., & Colangelo, A. (1996). Glucocorticoids differentially increase nerve growth factor and basic fibroblast growth factor expression in the rat brain. *J Neurosci*, *16*(6), 2141-2148.
- Muller, D. L., & Unterwald, E. M. (2004). In vivo regulation of extracellular signal-regulated protein kinase (ERK) and protein kinase B (Akt) phosphorylation by acute and chronic morphine. *J Pharmacol Exp Ther*, *310*(2), 774-782. doi: 10.1124/jpet.104.066548
- Nagata, A., Suzuki, Y., Igarashi, M., Eguchi, N., Toh, H., Urade, Y., & Hayaishi, O. (1991). Human brain prostaglandin D synthase has been evolutionarily differentiated from lipophilic-ligand carrier proteins. *Proc Natl Acad Sci U S A*, *88*(9), 4020-4024.
- Nagata, K., & Hirai, H. (2003). The second PGD(2) receptor CRTH2: structure, properties, and functions in leukocytes. *Prostaglandins Leukot Essent Fatty Acids*, *69*(2-3), 169-177.
- Nogawa, S., Zhang, F., Ross, M. E., & Iadecola, C. (1997). Cyclo-oxygenase-2 gene expression in neurons contributes to ischemic brain damage. *J Neurosci*, *17*(8), 2746-2755.
- Pervaiz, S., & Brew, K. (1987). Homology and structure-function correlations between alpha 1-acid glycoprotein and serum retinol-binding protein and its relatives. *FASEB J*, *1*(3), 209-214.
- Saleem, S., Shah, Z. A., Urade, Y., & Dore, S. (2009). Lipocalin-prostaglandin D synthase is a critical beneficial factor in transient and permanent focal cerebral ischemia. *Neuroscience*, *160*(1), 248-254. doi: 10.1016/j.neuroscience.2009.02.039
- Smith, W. L., Marnett, L. J., & DeWitt, D. L. (1991). Prostaglandin and thromboxane biosynthesis. *Pharmacol Ther*, *49*(3), 153-179.
- Sweatt, J. D. (2004). Mitogen-activated protein kinases in synaptic plasticity and memory. *Curr Opin Neurobiol*, *14*(3), 311-317. doi: 10.1016/j.conb.2004.04.001
- Tanaka, T., Urade, Y., Kimura, H., Eguchi, N., Nishikawa, A., & Hayaishi, O. (1997). Lipocalin-type prostaglandin D synthase (beta-trace) is a newly recognized type of retinoid transporter. *J Biol Chem*, *272*(25), 15789-15795.
- Taniguchi, H., Mohri, I., Okabe-Arahoru, H., Aritake, K., Wada, K., Kanekiyo, T., . . . Taniike, M. (2007). Prostaglandin D2 protects neonatal mouse brain from hypoxic

ischemic injury. *J Neurosci*, 27(16), 4303-4312. doi: 10.1523/JNEUROSCI.0321-07.2007

Taniguchi, H., Mohri, I., Okabe-Arahoru, H., Kanekiyo, T., Kagitani-Shimono, K., Wada, K., . . . Taniike, M. (2007). Early induction of neuronal lipocalin-type prostaglandin D synthase after hypoxic-ischemic injury in developing brains. *Neurosci Lett*, 420(1), 39-44. doi: 10.1016/j.neulet.2007.04.016

Tokudome, S., Sano, M., Shinmura, K., Matsubashi, T., Morizane, S., Moriyama, H., . . . Fukuda, K. (2009). Glucocorticoid protects rodent hearts from ischemia/reperfusion injury by activating lipocalin-type prostaglandin D synthase-derived PGD2 biosynthesis. *J Clin Invest*, 119(6), 1477-1488. doi: 10.1172/JCI37413

Urade, Y., & Hayaishi, O. (2000). Prostaglandin D synthase: structure and function. *Vitam Horm*, 58, 89-120.

Young, W., & Flamm, E. S. (1982). Effect of high-dose corticosteroid therapy on blood flow, evoked potentials, and extracellular calcium in experimental spinal injury. *J Neurosurg*, 57(5), 667-673. doi: 10.3171/jns.1982.57.5.0667

Yun, J. C., Ohman, K. P., Gill, J. R., Jr., & Keiser, H. (1991). Effects of prostaglandins, cAMP, and changes in cytosolic calcium on platelet aggregation induced by a thromboxane A2 mimic (U46619). *Can J Physiol Pharmacol*, 69(5), 599-604.

CHAPTER FIVE

GENERAL DISCUSSION

Certainly the habits generated by humans such as consuming healthy food, having a good exercise routine, smoking, or drinking induces a clear influence in an excellent or detrimental health life. However the environmental area can also play a significant role. One environmental area that has generated more interest is the environment to which the fetus is exposed to during development and how the fetus has to adapt to that environment that can compromise the development of some organs and ultimately increase the risk to certain conditions or diseases during adulthood. This interest began with the study presented by Barker, who shows that birth weight is inversely proportional to the risk of cardiovascular disease and hypertension in adulthood. This was the foundation for what is known today as the theory of developmental of health and disease (Barker, 2007). Different studies have been supporting this theory, where birth weight has a strong correlation with risk of developing cardiovascular (Barker & Osmond, 1986), metabolic (Hales & Ozanne, 2003), kidney, and lung (Gluckman, Hanson, Cooper, & Thornburg, 2008) disease among others. This low birth weight has been associated with some maternal perturbation as calorie and protein restriction (Lillicrop, Phillips, Jackson, Hanson, & Burdge, 2005), placental insufficiency (Pham et al., 2003), exposure to glucocorticoids (O'Sullivan et al., 2013) that induce a harsh environment and program adult onset disease. Our study demonstrated that perinatal hypoxia affected the development of the kidney by inducing aberrant expression of angiotensin II receptors

and accelerating the aging process of the kidney. Furthermore we demonstrated that perinatal hypoxia affected the development of the brain by increasing the vulnerability in neonates to hypoxic-ischemic brain injury, by repressing the expression of glucocorticoid receptors.

Effect of Hypoxia in Kidney Development

A hypoxia environment, on pregnant rats during their last week of pregnancy, affected the kidney organogenesis of the fetus. As has been mentioned, low birth weight is associated with programming diseases and perinatal hypoxia not just affecting the body weight of the E21 and P7 rats, but also affecting the kidney weight in an asymmetrical manner, suggesting that this adverse environment affects the development of the kidney. However for the 3M adult rats there was no difference between the control and the hypoxia treated group suggesting a “catch-up” process. Similar results were observed using maternal undernutrition and this acceleration during postnatal growth is a trigger for development of detrimental health life during adulthood (Lai et al., 2004).

As we reported, maternal hypoxia decreases the number of nephrons, oligonephronia, on P7 and 3M rat offspring. It is known that any adverse event occurring before completion of nephrogenesis likely compromises renal growth and produces a longer lasting effect on final renal function (Gray, Denton, Cullen-McEwen, Bertram, & Moritz, 2010). The nephrons are the structure in the kidney that induces the renal secretory and reabsorption functions; they filter the blood and also regulate the blood pressure among other functions, which means that a reduction in the number of nephrons could be detrimental. There is a hypothesis that states that a lower number of nephrons will decrease the surface area of filtration and this will lead to an increase in the

glomerular pressure and eventually will induce hypertension. A reduction number of nephrons due to uninephrectomy during the first 24 h after birth leads to increased blood pressure in adult rats (Woods, 1999). Additionally, evidence using animal models has shown that adverse environment during pregnancy as alcohol intake or a high salt diet induced oligonephronia in the offspring and increased their blood pressure during adulthood (Gray et al., 2010; Woods, Weeks, & Rasch, 2004), suggesting this, that programming hypertension due to maternal hypoxia could be observed due to the oligonephronia found in the offspring.

The oligonephronia observed during adulthood showed sexual dimorphism because, even though the effect was observed on both genders, the reduction on female (58%) was higher than the observed by the males (26%) suggesting that there is a gender-specific programming in the aberrant kidney development against the females. Interestingly, in a study using maternal protein restriction the male exhibited oligonephronia, while this event was not observed in female, demonstrating a sexual dimorphism effect against the males (Woods, Ingelfinger, Nyengaard, & Rasch, 2001; Woods, Ingelfinger, & Rasch, 2005). It will be interesting to evaluate the possible effects of adverse environment on gender hormones that could eventually result in sexual dimorphism of programming diseases. For example it is known that lung maturation for female fetuses during fetal development is faster than in male fetuses and this difference is apparently due to the presence of estrogen in the females that induces stimulatory effects (Patrone et al., 2003).

Otherwise, the Renin-Angiotensin System is both a circulating and tissue/organ specific hormonal system implicated in various physiological and pathophysiological

processes via the major peptide angiotensin II stimulating its specific AT₁R and AT₂R, which demonstrate opposite effects in many conditions (Dasgupta & Zhang, 2011; Sokol et al., 2004). In our study we evaluate the effect of maternal hypoxia environment in the expression of AT₁R and AT₂R on the offspring. This maternal insult has no effect on the protein abundance of both receptors in either fetal or neonatal rats as compared with normoxic animals. However, there was significant difference between both groups in the adult offspring for the AT₁R while no difference was found in the protein abundance of AT₂R. Moreover the maternal hypoxia also decreases the mRNA abundance of AT_{1a}R and AT_{1b}R in the kidney of the offspring. Nevertheless, a study using maternal nicotine showed that this maternal insult has no effect on the expression of AT₁R in rat kidney in three post-natal stages of the offspring, including adult, while the expression of AT₂R was decreased in all three post-natal stages (Mao et al., 2009). This may suggest that different maternal insults affect the organogenesis of the kidney in different ways.

Also, the RAS is of crucial importance during fetal development as angiotensin II acts as a potent growth factor in nephrogenesis (Woods et al., 2001). One role for angiotensin II during nephrogenesis is stimulating the branching of the ureteric bud by down-regulating the Sprouty 1 protein, which inhibits the receptor tyrosine kinase signaling, this effect will activate the *GDNF/c-Ret/Wnt11* pathway that will eventually lead to the branching of the ureteric bud and eventually the formation of the nephrons (Yosypiv, Boh, Spera, & El-Dahr, 2008). Recently, studies suggested that maternal insults suppresses the renin-angiotensin system of newborns, reduced the number of glomeruli and leads to hypertension during adulthood (Woods et al., 2001). Our study demonstrated no effect of maternal hypoxia in the RAS of fetus or neonates rats, but we

observed an aberrant development of the kidney due to a decrease on the number of nephrons on neonates and adult offspring. Nephrogenesis is controlled partly by RAS but there are other factors that are associated with the kidney development that could be affected by maternal hypoxia to induce the oligonephronia observed in our work. One possible mechanism that could be explored is Wnt4 that is needed for the differentiation of the metanephric mesenchyme into nephron epithelia (Stark, Vainio, Vassileva, & McMahon, 1994), or evaluate the effect of maternal hypoxia on the fibroblast growth factor-2 (Karavanova, Dove, Resau, & Perantoni, 1996) and on the transforming growth factor β -2 (Sanford et al., 1997), which are capable of inducing nephrogenesis. The retinoid receptors might be another potential target for maternal hypoxia because they mediate the remodeling process of epithelial cells associated with nephrogenesis (Batourina et al., 2001).

Finally, we observed that perinatal hypoxia decreased the number of glomeruli on offspring adult rats as compared with the normoxic group. The aging process in human kidney is associated with reduction of glomerular filtration rate, segmental glomerular sclerosis and reduction in the number of glomeruli (Epstein, 1996). Our findings not only suggest that perinatal adverse environment affected the kidneys in an aberrant way but also that this early hostile event could accelerate the kidney aging process.

Effect of Hypoxia on the Vulnerability of the Brain to Neonatal HIE

One of the advantages that perinatal studies offer is that they can give us some light of the pathogenesis of diseases and eventually lead us to develop some kind of treatment against those conditions. With this in mind we evaluate the effect of maternal

hypoxia in brain development of the rat offspring and how this insult affects the vulnerability of the brain against hypoxia-ischemic brain injury. Consistent with results reported in our kidney studies, maternal hypoxia affected the body weight of the fetus but also the weight of the brain in an asymmetrical pattern. Interestingly, when we compare the organ/body weight ratio for the brain and kidney, the ratio observed for the kidneys, which were exposed to maternal hypoxia, is lower than the control group; however the ratio observed for the brains that were exposed to hypoxia, was higher than for those brains of the normoxic group. This result may be in agreement with the concept that if the fetal body experiences a decrease in the level of delivered oxygen there will be a decrease of the amniotic fluid and a redistribution of fetal blood flow with a decrease in perfusion to the kidney and an increase of perfusion to the brain, heart and liver. The “catch-up” event was also observed in this study where the hypoxic fetus rats have a lower body and brain weight as compared to the normoxic fetuses but these differences were not observed in the P10 pups of both groups, and as mentioned before this could have long-term detrimental effects for the hypoxic neonates.

In chapter three we reported that maternal hypoxia increases the vulnerability of the brain toward neonatal hypoxic-ischemic encephalopathy by a mechanism that involved the repressing of the expression of glucocorticoid receptors. In the following section we will be discussing other possible mechanisms that could increase the vulnerability of the brain to HIE.

Mechanisms That Could be Involved in Fetal Stress-Mediated Increase in The Susceptibility of Neonatal HIE

The renin-angiotensin system could be a target for maternal insult where the

susceptibility of neonatal toward HIE is increased by reprogramming the expression patterns of the RAS in the brain (Mao et al., 2009). The action of the RAS is by the activation of AT₁R and AT₂R by angiotensin II, and it is known that RAS is a circulating and tissue/organ specific hormonal system implicated in various physiological and pathophysiological processes (Dasgupta & Zhang, 2011; Shi, Mao, Xu, & Zhang, 2010; Sokol et al., 2004). In the brain the presence of both AT₁R and AT₂R can be found during different developmental stages with different expression patterns and signaling pathways. The importance of RAS in the development and evolution of cerebrovascular diseases has been reported in clinical trials and experimental studies with mature brains. LIFE and MOSES are examples of clinical trials that have been shown that by blocking the RAS it demonstrated neuroprotection with the prevention of first of recurrent strokes in high-risk populations, independent of its blood pressure-lowering effect (Lindholm et al., 2002; Schrader et al., 2005). Anti-apoptotic, anti-inflammatory, anti-oxidant effects can be observed in studies with AT₁R antagonist which improve cerebral perfusion, demonstrating vascular-dependent and -independent neuroprotection in acute stroke (Ando, Zhou, Macova, Imboden, & Saavedra, 2004; Dai, Funk, Herdegen, Unger, & Culman, 1999; Lou et al., 2004; Zhou, Ando, Macova, Dou, & Saavedra, 2005). Although very little is known about the role of AT₂R in neurological pathophysiology, evidence has been found that demonstrated the beneficial effect of AT₂R in a variety of pathologies including various neurological conditions. The neuroprotective role of AT₂R was suggested when this receptor was up regulated in the brain, mostly in the ischemic area during stroke (Mogi et al., 2006), besides increased activation of AT₂R may be responsible for some neuroprotective effects of AT₁R antagonism (Li et al., 2005). In

vitro stimulation of AT₂R promotes intense neurite outgrowth, which can be antagonized by PD123319 (Laflamme, Gasparo, Gallo, Payet, & Gallo-Payet, 1996). Additionally, in a rat model of stroke, a direct stimulation of AT₂R with CPG42112 induced a neuroprotective effect that was beyond blood pressure regulation (McCarthy, Vinh, Callaway, & Widdop, 2009). The underlying mechanisms of AT₂R in neuroprotection remain to be elucidated. Some studies indicated that it might be related to its complicated interaction with AT₁R in apoptotic modulation, neuronal regeneration and vasodilation in ischemic regions following stroke (Jones, Vinh, McCarthy, Gaspari, & Widdop, 2008; Saavedra, Benicky, & Zhou, 2006).

Fetal stress may also reprogram expression patterns of matrix metalloproteinase in the neonatal brain, which contribute to the enhanced vulnerability of HIE. MMPs belong to a family of zinc-dependent proteases that exert pronounced effects in the ECM turnover. These enzymes remodel almost all components of the matrix and play an essential role in cell signaling regulation, cell survival and cell death. MMPs, especially MMP-2, MMP-3 and MMP-9, may target the extracellular matrix of blood vessels, basal lamina, and tight junctions in endothelial cells, increase the permeability of the blood-brain barrier in neuroinflammation due to hypoxia-ischemia, multiple sclerosis and CNS infection, which can result in cytotoxic and vasogenic edema, promote hemorrhagic transformation, induce apoptosis of neurons and oligodendrocytes (Cunningham, Wetzel, & Rosenberg, 2005; Rosenberg, 2009). However, in later stage of such pathology, MMPs play critical roles in tissue repair and remodeling process via inducing angiogenesis and neurogenesis. Growing evidence suggests that overly up-regulated activity/expression of MMPs, particularly MMP-2 and MMP-9, is deleterious in the acute phase of stroke.

Inhibition of MMPs in the acute phase may reduce the damage to BBB (Gasche, Copin, Sugawara, Fujimura, & Chan, 2001). There is a report indicating decreased damage to BBB and reduced infarct size in a focal ischemic MMP-9 knockout model (Asahi et al., 2001). More importantly, a recent study in a neonatal rat HIE model revealed that early inhibition of MMPs conferred acute and long-term beneficial effects via reducing tight junction proteins degradation, attenuating the permeability of BBB, improving brain edema, and preventing brain atrophy (W. Chen et al., 2009). Fetal hypoxia reprograms expression patterns of MMPs in the heart and brain and increases activities/expressions of both MMP-2 and MMP-9 in the neonatal brain (Tong et al., 2010; Tong, Xue, Li, & Zhang, 2011; Tong & Zhang, 2012). Considering the evident detrimental effects of MMPs in the acute stroke models and other neurological pathophysiologies, it is plausible that altered expression patterns of MMPs by prenatal stress are other important mediators in programming of ischemic-sensitive phenotype and increased susceptibility of HIE in the neonatal brain.

Epigenetic Mechanisms in Fetal Programming

In our study we found that the expression of the glucocorticoid receptors was repressed due to hypermethylation in the promoter region of the GR due to maternal hypoxia. One of the important adaptive mechanisms that the human body could be evoked to react to some adverse environments is through epigenetic modification of gene expression patterns. The fetal developmental stage is the most critical period for the human being because in the uterus the fetus can be exposed to inadequate or inappropriate environments that could be chemical/ nutritional or non-chemical. These epigenetic changes could be associated with conditions or diseases during adulthood.

The fetus is a critical developmental stage in which different events occur in a way to induce repression or activation of gene transcription via epigenetic mechanisms (M. Chen & Zhang, 2011). Brain-derived neurotrophic factor plays a vital role in the brain development. An epidemiologic study in adolescents whose mothers smoked during pregnancy revealed that prenatal nicotine exposure increases DNA methylation of the BDNF-6 exon, and this may lead to changes in the plasticity and development of the brain (Toledo-Rodriguez et al., 2010). A recent study evaluated the DNA methylation patterns of the genes coding GR, 11b-HSD2, neuronatin and reelin in hippocampus of the offspring rats from pregnant animals that had been treated with a deficient methyl donor diet. Though the behavior differences were demonstrated between MDD and the control groups, the DNA methylation patterns of these genes were not altered (Konycheva et al., 2011). However, it has been shown that maternal stress of pregnant rats during gestational days 12–16 increases the levels of DNA methylation in frontal cortex and hippocampus in offspring, which is associated with behavioral changes in offspring rats (Mychasiuk, Ilnytsky, Kovalchuk, Kolb, & Gibb, 2011). Additionally, it has been found that fetal exposure to bisphenol A (BPA), a xenoestrogen, induces changes in DNA methylation patterns in the 2500 NotI loci, suggesting that the maternal BPA exposure may also exert some programming effects on brain development (Yaoi et al., 2008).

Recently, there has been increased interest to evaluate the effect of maternal environment on other epigenetic modifications that could lead to programming diseases. Synthetic glucocorticoids are used during pregnancy to support the maturation of fetal organs. Though the exposure to sGC during fetal development has resulted detrimental, inducing endocrine and metabolic abnormalities in animal offspring. Moreover it has

been reported that fetal exposure to sGC resulted in substantial differences in the acetylation of histone h3 lysine 9, in specific gene promoters (Crudo et al., 2013). In a rat model of low protein diets it was demonstrated that this maternal environment repressed the expression of the liver X receptor α and also decreased the acetylation of H3K9 and H3K14 which surrounds the transcriptional start site of $Lxr\alpha$. The down regulation of this receptor leads to impaired glucose tolerance in the offspring (Vo, Revesz, Sohi, Ma, & Hardy, 2013). Maternal smoking during fetal development increases the risk of overweight to the offspring (Weng, Redsell, Swift, Yang, & Glazebrook, 2012). In a rat model, elevated levels of triglycerides were found in the circulation and liver of the offspring, associated with maternal nicotine. The synthesis of triglycerides is catalyzed by the fatty acid synthase, which was up-regulated due to maternal nicotine. The FAS is regulated by $Lxr\alpha$, the expression of this receptor was also up-regulated and associated with an hyperacetylation of H3K9 and H3K14 which are close to the *FAS* promoter (Ma, Nicholson, Wong, Holloway, & Hardy, 2014).

Another fetal stress associated with histone modification is maternal caffeine intake. This type of insult inhibited the production of adrenal corticosterone in fetus. The expression of StAR, which is an enzyme associated with corticosterone production, was down-regulated in the rat fetal adrenal due to maternal caffeine consumption (Ping et al., 2014). This stress also affects the histone acetylation of the SF-1 promoter, which is a key transcription factor for StAR. The decreased in the acetylation levels of H3K9 and H3K14 in the promoter region of SF-1 was associated with the down-regulation of StAR.

Potential Neuroprotective Mechanism Against HIE

In chapter three we suggested a neuroprotective mechanism for glucocorticoid receptors against HIE. Our study implied that dexamethasone induced L-PGDS expression, preferentially promoted PGD₂ biosynthesis in the developing brain, which acted as a local mediator in an autocrine and/or paracrine manner to confer protective effects via chiefly interaction with DP₁ receptor. Another finding of that study is; that pERK-44 acted as the major downstream effector in dexamethasone pretreatment mediated neuroprotection in neonatal HI brain injury (Figure 1). Apoptosis is one of the cell death mechanism found on HI brain injury. It is well known that activation of ERK/MAPK is associated with cell survival by increasing the expression of Bcl-2, which repress the activity of caspase 3 resulting in a decrease of apoptosis level. Further experimentation into this mechanism in our HIE model will help to increase our knowledge on the neuroprotection mechanism of dexamethasone. Also in this section we are going to discuss some other potential neuroprotective mechanisms. A possible neuroprotective role against HIE has been suggested for the delta opioid receptors. In a model of asphyxia cardiac arrest, the activation of DOR by the specific agonist [d-Ala², d-Leu⁵] enkephalin induced neuronal survival, neuroprotective effect that was reversed by Naltrindole, an antagonist for DOR (Gao et al., 2010). DADLE also show neuroprotection by inducing neuron survival in astrocytes of the hippocampal CA1 region after global cerebral ischemia (Duan et al., 2011). During an event of HIE the ATP-dependent Na⁺-K⁺ pumps are impaired. This may result in an influx of Na⁺ and a leak of K⁺, which is detrimental for the cell.

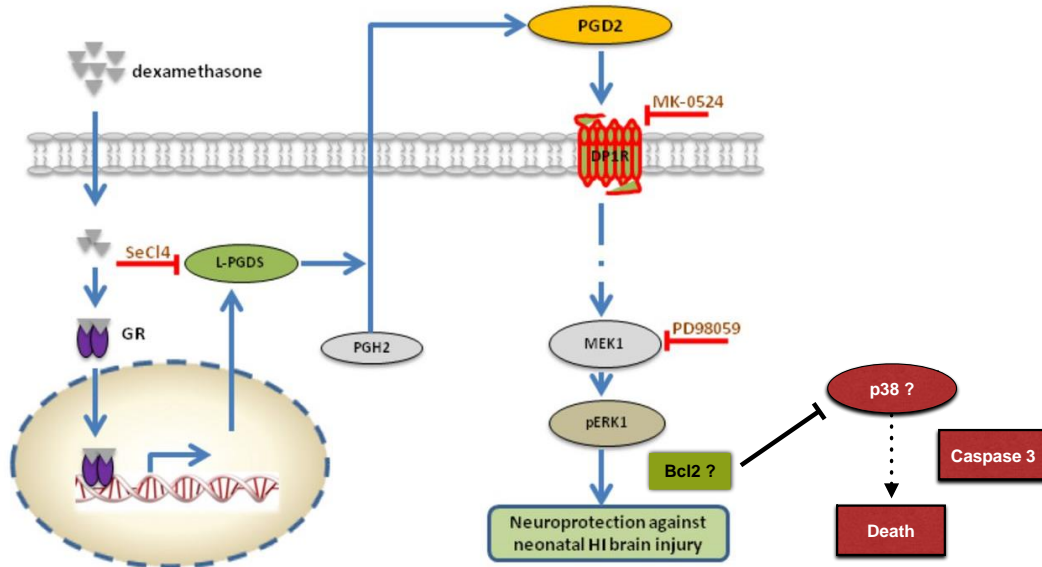


Figure 1. Proposed signaling pathway in dexamethasone pretreatment induced neuroprotection against neonatal hypoxic-ischemic brain injury. GR indicates glucocorticoids receptor; L-PGDS indicates lipocalin-type prostaglandin D synthase; PGH₂ indicates prostaglandin H₂; PGD₂ indicates prostaglandin D₂; DP₁ indicates D prostanoid; MEK1 indicates mitogen-activated kinase/ERK kinase 1; pERK-42/44 indicates phosphorylated extracellular signal regulated kinase 42/44; SeCl₄ indicates selenium chloride, a selective inhibitor of lipocalin-type prostaglandin D synthase (L-PGDS); PD98059, selective inhibitor of MEK1; MK-0524, a selective antagonist for prostaglandin D₂ receptor (DP₁); HI indicates hypoxic-ischemic; Bcl-2 indicates B-cell lymphoma 2; p38 indicates.

The activation of DOR in an anoxic model decreased the K⁺ leakage and also the Na⁺ influx in cortical neurons (Chao, Balboni, Lazarus, Salvadori, & Xia, 2009). Moreover, HIE is characterized by mitochondrial dysfunction. In a study with cultured cortical neurons, sodium azide inhibited the mitochondrial respiratory chain, inducing brain injury that was reduced by the activation of DOR. This neuroprotective effect was inhibited by NTI (Zhu et al., 2009).

Another possible neuroprotective mechanism against HIE is the cannabinoid receptor type 1. The CB1 receptors are G protein-protein coupled receptors that could be located in the basal ganglia, amygdala, cerebellum, hippocampus, cerebral cortex and in the hypothalamus as well as in tissues from other organs (Galiegue et al., 1995). Another detrimental effect of HIE is the generation of nitric oxide by the nitric oxide synthase. The activation of CB1 in brain microglial cells of rats, inhibit the NO production (Cabral, Harmon, & Carlisle, 2001). Rat brain slices were exposed to oxygen-glucose deprivation for 30 min that resulted in an increase of glutamate and TNF- α . By the activation of CB1 neuroprotective effect was observed, the production of glutamate and TNF- α was inhibited, however, when an antagonist of CB1 was used the neuroprotective effect was diminished, suggesting that the neuroprotective effect was mediated by CB1 (Fernandez-Lopez et al., 2006). The activation of CB1 also reduced the infarct volume after global and focal cerebral ischemia and protected cultured cerebral cortical neurons that were exposed to hypoxia and glucose deficiency (Nagayama et al., 1999).

Conclusion

The fetal development period is still the most critical experience for the human being. In this work we demonstrated how, during this event, maternal stress could lead to some aberrant organogenesis in the fetus and eventually programming the offspring to an increase risk of disease vulnerability. We demonstrated in this work that maternal hypoxia, one of the most common adverse environments experienced by fetus, affects the fetal growth and development of the kidney and the brain of the offspring. This IUGR induces low weight birth on the neonates a common effect that has been found in the process of programming diseases. The structure of the kidney was affected by the maternal insult, where a lower number of glomeruli were observed for the hypoxic offspring where females were more affected than the males during adulthood, resulting in a sex dimorphism effect. Additionally, at molecular level maternal hypoxia induced an aberrant development by affecting the normal expression of AT1R. These observations suggested that fetal hypoxia, cause an aberrant development of the kidney and accelerate the aging process during postnatal development that could increase the risk to cardiovascular diseases during adulthood.

The brain development was also affected by this maternal insult where the vulnerability toward HIE was increased due to this harmful fetal environment. Moreover it was demonstrated that maternal hypoxia affected the expression of the glucocorticoid receptors for which a neuroprotective role against neonate HIE brain injury was found. The down-regulation of the GR was associated to a hypermethylation event observed in the promoter region of the GR gene, suggesting that maternal environment could affect the organogenesis of the brain by inducing epigenetic changes in the fetus genome. Finally the mechanism of the neuroprotective action of GR was evaluated, where the

activation of the MAPK/ERK pathway, by dexamethasone, was observed. Moreover, dexamethasone increases the expression of LPGDS and the levels of PDG₂, where the inhibition of LPGDS and blockade of DP1 receptor represses the neuroprotective role of DEX. These results suggest that LPGDS/PGD cascade plays a significant role in the neuroprotective mechanism of GR.

These novel findings provide evidence that maternal environment could affect the fetal development including the organogenesis of the brain and the kidney. The possibility that maternal hypoxia increased the risk to HIE provide a mechanistic understanding that could be evaluated in human. The findings that the repression of the GR was mediated by this fetal stress and that glucocorticoid activates the MAPK/ERK pathway with LPGDS/PGD cascade playing a significant role, provides a novel mechanism in the understanding of HIE at molecular level and suggests new insight for development of new therapeutic strategies against neonate HIE.

References

- Ando, H., Zhou, J., Macova, M., Imboden, H., & Saavedra, J. M. (2004). Angiotensin II AT1 receptor blockade reverses pathological hypertrophy and inflammation in brain microvessels of spontaneously hypertensive rats. *Stroke*, *35*(7), 1726-1731. doi: 10.1161/01.STR.0000129788.26346.18
- Asahi, M., Wang, X., Mori, T., Sumii, T., Jung, J. C., Moskowitz, M. A., . . . Lo, E. H. (2001). Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J Neurosci*, *21*(19), 7724-7732.
- Barker, D. J. (2007). The origins of the developmental origins theory. *J Intern Med*, *261*(5), 412-417. doi: 10.1111/j.1365-2796.2007.01809.x
- Barker, D. J., & Osmond, C. (1986). Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet*, *1*(8489), 1077-1081.
- Batourina, E., Gim, S., Bello, N., Shy, M., Clagett-Dame, M., Srinivas, S., . . . Mendelsohn, C. (2001). Vitamin A controls epithelial/mesenchymal interactions through Ret expression. *Nat Genet*, *27*(1), 74-78. doi: 10.1038/83792
- Cabral, G. A., Harmon, K. N., & Carlisle, S. J. (2001). Cannabinoid-mediated inhibition of inducible nitric oxide production by rat microglial cells: evidence for CB1 receptor participation. *Adv Exp Med Biol*, *493*, 207-214. doi: 10.1007/0-306-47611-8_24
- Chao, D., Balboni, G., Lazarus, L. H., Salvadori, S., & Xia, Y. (2009). Na⁺ mechanism of delta-opioid receptor induced protection from anoxic K⁺ leakage in the cortex. *Cell Mol Life Sci*, *66*(6), 1105-1115. doi: 10.1007/s00018-009-8759-5
- Chen, M., & Zhang, L. (2011). Epigenetic mechanisms in developmental programming of adult disease. *Drug Discov Today*, *16*(23-24), 1007-1018. doi: 10.1016/j.drudis.2011.09.008
- Chen, W., Hartman, R., Ayer, R., Marcantonio, S., Kamper, J., Tang, J., & Zhang, J. H. (2009). Matrix metalloproteinases inhibition provides neuroprotection against hypoxia-ischemia in the developing brain. *J Neurochem*, *111*(3), 726-736. doi: 10.1111/j.1471-4159.2009.06362.x
- Crudo, A., Suderman, M., Moisiadis, V. G., Petropoulos, S., Kostaki, A., Hallett, M., . . . Matthews, S. G. (2013). Glucocorticoid programming of the fetal male hippocampal epigenome. *Endocrinology*, *154*(3), 1168-1180. doi: 10.1210/en.2012-1980
- Cunningham, L. A., Wetzel, M., & Rosenberg, G. A. (2005). Multiple roles for MMPs and TIMPs in cerebral ischemia. *Glia*, *50*(4), 329-339. doi: 10.1002/glia.20169

- Dai, W. J., Funk, A., Herdegen, T., Unger, T., & Culman, J. (1999). Blockade of central angiotensin AT(1) receptors improves neurological outcome and reduces expression of AP-1 transcription factors after focal brain ischemia in rats. *Stroke*, *30*(11), 2391-2398; discussion 2398-2399.
- Dasgupta, C., & Zhang, L. (2011). Angiotensin II receptors and drug discovery in cardiovascular disease. *Drug Discov Today*, *16*(1-2), 22-34. doi: 10.1016/j.drudis.2010.11.016
- Duan, Y. L., Wang, S. Y., Zeng, Q. W., Su, D. S., Li, W., Wang, X. R., & Zhao, Z. (2011). Astroglial reaction to delta opioid peptide [D-Ala2, D-Leu5] enkephalin confers neuroprotection against global ischemia in the adult rat hippocampus. *Neuroscience*, *192*, 81-90. doi: 10.1016/j.neuroscience.2011.06.067
- Epstein, M. (1996). Aging and the kidney. *J Am Soc Nephrol*, *7*(8), 1106-1122.
- Fernandez-Lopez, D., Martinez-Orgado, J., Nunez, E., Romero, J., Lorenzo, P., Moro, M. A., & Lizasoain, I. (2006). Characterization of the neuroprotective effect of the cannabinoid agonist WIN-55212 in an in vitro model of hypoxic-ischemic brain damage in newborn rats. *Pediatr Res*, *60*(2), 169-173. doi: 10.1203/01.pdr.0000228839.00122.6c
- Galiegue, S., Mary, S., Marchand, J., Dussossoy, D., Carriere, D., Carayon, P., . . . Casellas, P. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur J Biochem*, *232*(1), 54-61.
- Gao, C. J., Li, J. P., Wang, W., Lu, B. C., Niu, L., Zhu, C., . . . Li, Y. Q. (2010). Effects of intracerebroventricular application of the delta opioid receptor agonist [D-Ala2, D-Leu5] enkephalin on neurological recovery following asphyxial cardiac arrest in rats. *Neuroscience*, *168*(2), 531-542. doi: 10.1016/j.neuroscience.2010.02.025
- Gasche, Y., Copin, J. C., Sugawara, T., Fujimura, M., & Chan, P. H. (2001). Matrix metalloproteinase inhibition prevents oxidative stress-associated blood-brain barrier disruption after transient focal cerebral ischemia. *J Cereb Blood Flow Metab*, *21*(12), 1393-1400. doi: 10.1097/00004647-200112000-00003
- Gluckman, P. D., Hanson, M. A., Cooper, C., & Thornburg, K. L. (2008). Effect of in utero and early-life conditions on adult health and disease. *N Engl J Med*, *359*(1), 61-73. doi: 10.1056/NEJMra0708473
- Gray, S. P., Denton, K. M., Cullen-McEwen, L., Bertram, J. F., & Moritz, K. M. (2010). Prenatal exposure to alcohol reduces nephron number and raises blood pressure in progeny. *J Am Soc Nephrol*, *21*(11), 1891-1902. doi: 10.1681/ASN.2010040368

- Hales, C. N., & Ozanne, S. E. (2003). For debate: Fetal and early postnatal growth restriction lead to diabetes, the metabolic syndrome and renal failure. *Diabetologia*, *46*(7), 1013-1019. doi: 10.1007/s00125-003-1131-7
- Jones, E. S., Vinh, A., McCarthy, C. A., Gaspari, T. A., & Widdop, R. E. (2008). AT2 receptors: functional relevance in cardiovascular disease. *Pharmacol Ther*, *120*(3), 292-316. doi: 10.1016/j.pharmthera.2008.08.009
- Karavanova, I. D., Dove, L. F., Resau, J. H., & Perantoni, A. O. (1996). Conditioned medium from a rat ureteric bud cell line in combination with bFGF induces complete differentiation of isolated metanephric mesenchyme. *Development*, *122*(12), 4159-4167.
- Konycheva, G., Dziadek, M. A., Ferguson, L. R., Krageloh, C. U., Coolen, M. W., Davison, M., & Breier, B. H. (2011). Dietary methyl donor deficiency during pregnancy in rats shapes learning and anxiety in offspring. *Nutr Res*, *31*(10), 790-804. doi: 10.1016/j.nutres.2011.09.015
- Laflamme, L., Gasparo, M., Gallo, J. M., Payet, M. D., & Gallo-Payet, N. (1996). Angiotensin II induction of neurite outgrowth by AT2 receptors in NG108-15 cells. Effect counteracted by the AT1 receptors. *J Biol Chem*, *271*(37), 22729-22735.
- Lai, K. N., Chan, L. Y., Tang, S. C., Tsang, A. W., Li, F. F., Lam, M. F., . . . Leung, J. C. (2004). Mesangial expression of angiotensin II receptor in IgA nephropathy and its regulation by polymeric IgA1. *Kidney Int*, *66*(4), 1403-1416. doi: 10.1111/j.1523-1755.2004.00874.x
- Li, J., Culman, J., Hortnagl, H., Zhao, Y., Gerova, N., Timm, M., . . . Unger, T. (2005). Angiotensin AT2 receptor protects against cerebral ischemia-induced neuronal injury. *FASEB J*, *19*(6), 617-619. doi: 10.1096/fj.04-2960fje
- Lillicrop, K. A., Phillips, E. S., Jackson, A. A., Hanson, M. A., & Burdge, G. C. (2005). Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr*, *135*(6), 1382-1386.
- Lindholm, L. H., Ibsen, H., Dahlof, B., Devereux, R. B., Beevers, G., de Faire, U., . . . Group, L. S. (2002). Cardiovascular morbidity and mortality in patients with diabetes in the Losartan Intervention For Endpoint reduction in hypertension study (LIFE): a randomised trial against atenolol. *Lancet*, *359*(9311), 1004-1010. doi: 10.1016/S0140-6736(02)08090-X
- Lou, M., Blume, A., Zhao, Y., Gohlke, P., Deuschl, G., Herdegen, T., & Culman, J. (2004). Sustained blockade of brain AT1 receptors before and after focal cerebral ischemia alleviates neurologic deficits and reduces neuronal injury, apoptosis, and

inflammatory responses in the rat. *J Cereb Blood Flow Metab*, 24(5), 536-547. doi: 10.1097/00004647-200405000-00008

- Ma, N., Nicholson, C. J., Wong, M., Holloway, A. C., & Hardy, D. B. (2014). Fetal and neonatal exposure to nicotine leads to augmented hepatic and circulating triglycerides in adult male offspring due to increased expression of fatty acid synthase. *Toxicol Appl Pharmacol*, 275(1), 1-11. doi: 10.1016/j.taap.2013.12.010
- Mao, C., Wu, J., Xiao, D., Lv, J., Ding, Y., Xu, Z., & Zhang, L. (2009). The effect of fetal and neonatal nicotine exposure on renal development of AT(1) and AT(2) receptors. *Reprod Toxicol*, 27(2), 149-154. doi: 10.1016/j.reprotox.2009.01.012
- McCarthy, C. A., Vinh, A., Callaway, J. K., & Widdop, R. E. (2009). Angiotensin AT2 receptor stimulation causes neuroprotection in a conscious rat model of stroke. *Stroke*, 40(4), 1482-1489. doi: 10.1161/STROKEAHA.108.531509
- Mogi, M., Li, J. M., Iwanami, J., Min, L. J., Tsukuda, K., Iwai, M., & Horiuchi, M. (2006). Angiotensin II type-2 receptor stimulation prevents neural damage by transcriptional activation of methyl methanesulfonate sensitive 2. *Hypertension*, 48(1), 141-148. doi: 10.1161/01.HYP.0000229648.67883.f9
- Mychasiuk, R., Ilnytskyi, S., Kovalchuk, O., Kolb, B., & Gibb, R. (2011). Intensity matters: brain, behaviour and the epigenome of prenatally stressed rats. *Neuroscience*, 180, 105-110. doi: 10.1016/j.neuroscience.2011.02.026
- Nagayama, T., Sinor, A. D., Simon, R. P., Chen, J., Graham, S. H., Jin, K., & Greenberg, D. A. (1999). Cannabinoids and neuroprotection in global and focal cerebral ischemia and in neuronal cultures. *J Neurosci*, 19(8), 2987-2995.
- O'Sullivan, L., Cuffe, J. S., Paravicini, T. M., Campbell, S., Dickinson, H., Singh, R. R., . . . Moritz, K. M. (2013). Prenatal exposure to dexamethasone in the mouse alters cardiac growth patterns and increases pulse pressure in aged male offspring. *PLoS One*, 8(7), e69149. doi: 10.1371/journal.pone.0069149
- Patrone, C., Cassel, T. N., Pettersson, K., Piao, Y. S., Cheng, G., Ciana, P., . . . Nord, M. (2003). Regulation of postnatal lung development and homeostasis by estrogen receptor beta. *Mol Cell Biol*, 23(23), 8542-8552.
- Pham, T. D., MacLennan, N. K., Chiu, C. T., Laksana, G. S., Hsu, J. L., & Lane, R. H. (2003). Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. *Am J Physiol Regul Integr Comp Physiol*, 285(5), R962-970. doi: 10.1152/ajpregu.00201.2003
- Ping, J., Wang, J. F., Liu, L., Yan, Y. E., Liu, F., Lei, Y. Y., & Wang, H. (2014). Prenatal caffeine ingestion induces aberrant DNA methylation and histone acetylation of steroidogenic factor 1 and inhibits fetal adrenal steroidogenesis. *Toxicology*. doi: 10.1016/j.tox.2014.03.011

- Rosenberg, G. A. (2009). Matrix metalloproteinases and their multiple roles in neurodegenerative diseases. *Lancet Neurol*, 8(2), 205-216. doi: 10.1016/S1474-4422(09)70016-X
- Saavedra, J. M., Benicky, J., & Zhou, J. (2006). Mechanisms of the Anti-Ischemic Effect of Angiotensin II AT(1) Receptor Antagonists in the Brain. *Cell Mol Neurobiol*, 26(7-8), 1099-1111. doi: 10.1007/s10571-006-9009-0
- Sanford, L. P., Ormsby, I., Gittenberger-de Groot, A. C., Sariola, H., Friedman, R., Boivin, G. P., . . . Doetschman, T. (1997). TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. *Development*, 124(13), 2659-2670.
- Schrader, J., Luders, S., Kulschewski, A., Hammersen, F., Plate, K., Berger, J., . . . Group, M. S. (2005). Morbidity and Mortality After Stroke, Eprosartan Compared with Nitrendipine for Secondary Prevention: principal results of a prospective randomized controlled study (MOSES). *Stroke*, 36(6), 1218-1226. doi: 10.1161/01.STR.0000166048.35740.a9
- Shi, L., Mao, C., Xu, Z., & Zhang, L. (2010). Angiotensin-converting enzymes and drug discovery in cardiovascular diseases. *Drug Discov Today*, 15(9-10), 332-341. doi: 10.1016/j.drudis.2010.02.003
- Sokol, S. I., Portnay, E. L., Curtis, J. P., Nelson, M. A., Hebert, P. R., Setaro, J. F., & Foody, J. M. (2004). Modulation of the renin-angiotensin-aldosterone system for the secondary prevention of stroke. *Neurology*, 63(2), 208-213.
- Stark, K., Vainio, S., Vassileva, G., & McMahon, A. P. (1994). Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature*, 372(6507), 679-683. doi: 10.1038/372679a0
- Toledo-Rodriguez, M., Lotfipour, S., Leonard, G., Perron, M., Richer, L., Veillette, S., . . . Paus, T. (2010). Maternal smoking during pregnancy is associated with epigenetic modifications of the brain-derived neurotrophic factor-6 exon in adolescent offspring. *Am J Med Genet B Neuropsychiatr Genet*, 153B(7), 1350-1354. doi: 10.1002/ajmg.b.31109
- Tong, W., Chen, W., Ostrowski, R. P., Ma, Q., Souvenir, R., Zhang, L., . . . Tang, J. (2010). Maternal hypoxia increases the activity of MMPs and decreases the expression of TIMPs in the brain of neonatal rats. *Dev Neurobiol*, 70(3), 182-194. doi: 10.1002/dneu.20770
- Tong, W., Xue, Q., Li, Y., & Zhang, L. (2011). Maternal hypoxia alters matrix metalloproteinase expression patterns and causes cardiac remodeling in fetal and neonatal rats. *Am J Physiol Heart Circ Physiol*, 301(5), H2113-2121. doi: 10.1152/ajpheart.00356.2011

- Tong, W., & Zhang, L. (2012). Fetal hypoxia and programming of matrix metalloproteinases. *Drug Discov Today*, 17(3-4), 124-134. doi: 10.1016/j.drudis.2011.09.011
- Vo, T. X., Revesz, A., Sohi, G., Ma, N., & Hardy, D. B. (2013). Maternal protein restriction leads to enhanced hepatic gluconeogenic gene expression in adult male rat offspring due to impaired expression of the liver X receptor. *J Endocrinol*, 218(1), 85-97. doi: 10.1530/JOE-13-0055
- Weng, S. F., Redsell, S. A., Swift, J. A., Yang, M., & Glazebrook, C. P. (2012). Systematic review and meta-analyses of risk factors for childhood overweight identifiable during infancy. *Arch Dis Child*, 97(12), 1019-1026. doi: 10.1136/archdischild-2012-302263
- Woods, L. L. (1999). Neonatal uninephrectomy causes hypertension in adult rats. *Am J Physiol*, 276(4 Pt 2), R974-978.
- Woods, L. L., Ingelfinger, J. R., Nyengaard, J. R., & Rasch, R. (2001). Maternal protein restriction suppresses the newborn renin-angiotensin system and programs adult hypertension in rats. *Pediatr Res*, 49(4), 460-467. doi: 10.1203/00006450-200104000-00005
- Woods, L. L., Ingelfinger, J. R., & Rasch, R. (2005). Modest maternal protein restriction fails to program adult hypertension in female rats. *Am J Physiol Regul Integr Comp Physiol*, 289(4), R1131-1136. doi: 10.1152/ajpregu.00037.2003
- Woods, L. L., Weeks, D. A., & Rasch, R. (2004). Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney Int*, 65(4), 1339-1348. doi: 10.1111/j.1523-1755.2004.00511.x
- Yaoi, T., Itoh, K., Nakamura, K., Ogi, H., Fujiwara, Y., & Fushiki, S. (2008). Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol A. *Biochem Biophys Res Commun*, 376(3), 563-567. doi: 10.1016/j.bbrc.2008.09.028
- Yosypiv, I. V., Boh, M. K., Spera, M. A., & El-Dahr, S. S. (2008). Downregulation of Spry-1, an inhibitor of GDNF/Ret, causes angiotensin II-induced ureteric bud branching. *Kidney Int*, 74(10), 1287-1293. doi: 10.1038/ki.2008.378
- Zhou, J., Ando, H., Macova, M., Dou, J., & Saavedra, J. M. (2005). Angiotensin II AT1 receptor blockade abolishes brain microvascular inflammation and heat shock protein responses in hypertensive rats. *J Cereb Blood Flow Metab*, 25(7), 878-886. doi: 10.1038/sj.jcbfm.9600082

Zhu, M., Li, M. W., Tian, X. S., Ou, X. M., Zhu, C. Q., & Guo, J. C. (2009). Neuroprotective role of delta-opioid receptors against mitochondrial respiratory chain injury. *Brain Res*, *1252*, 183-191. doi: 10.1016/j.brainres.2008.11.030