PRENATAL STRESS-MEDIATED FETAL PROGRAMMING OF OBESITY IN DIET-INDUCED OBESE AND DIETARY RESISTANT RATS

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ABSTRACT

PRENATAL STRESS-MEDIATED FETAL PROGRAMMING OF OBESITY IN DIET-INDUCED OBESE AND DIETARY RESISTANT RATS

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Genotype, diet and environment or life style factors are known to be associated with the development of obesity and metabolic syndrome in adults. However, recent evidence suggests that prenatal factors could contribute to obesity as well. One of the prenatal factors implicated in the development of metabolic syndrome in the offspring is stress, which is known to increase circulating glucocorticoids during pregnancy. Prolonged exposure of the developing fetus to excess glucocorticoid levels results in long lasting neuroendocrine changes which predispose the offspring to obesity and other cardiovascular disorders. Considering the fact that 30% of today's maternal population is obese, it is also important to address the impact of prenatal stress in the background of maternal obesity. Hence, we used diet-induced obese (DIO) and dietary resistant (DR) rat model to explore the mechanisms underlying prenatal stress mediated fetal programming of obesity in DIO and DR rats.

Prenatal stress was associated with catch up growth and hyperinsulinemia in the DIO offspring. Although, prenatal stress reduced birth weight in the DR offspring, it did not result in any other adverse metabolic outcomes. Next, we investigated the role of stress axis hyperactivation in prenatal stress-induced metabolic programming in the DIO offspring. In the DIO rats, prenatal stress resulted in hyperactivation of stress axis marked by increased norepinephrine (NE) levels in the paraventricular nucleus in the

hypothalamus and increased corticotrophin releasing hormone levels in the median eminence. However, the serum corticosterone (CORT) levels were not altered in the prenatally stressed DIO and DR offspring. Despite, no change in circulating CORT levels, glucocorticoids might play a role in metabolic syndrome through increasing 11β-hydroxysteroid dehydrogenase enzyme (11βHSD1) in the target tissues. 11βHSD1 is highly expressed in metabolically active tissues like liver and adipose tissue and is involved in the intracellular generation of CORT by converting inactive 11-dehydroCORT to active CORT. Hence, we investigated the role of 11βHSD1 in the liver and adipose tissue in prenatal stress mediated metabolic programming. Prenatal stress significantly increased 11βHSD1 mRNA and protein expression in the visceral adipose tissue accompanied with hypertrophied adipocytes in the DIO offspring. There were no differences in 11βHSD1 expression in the liver suggesting prenatal stress results in tissue-specific programming of 11βHSD1 expression. Taken together, the results suggest that prenatal stress produces differential metabolic effects in DIO and DR rats. Further, 11βHSD1 could mediate the metabolic effects observed in the prenatally stressed offspring and thus may be a potential mechanism for fetal origins of obesity.

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11-DHC	11-Dehydrocorticosterone
11βHSD1	11β-hydroxysteroid dehydrogenase type 1 enzyme
11βHSD2	11β-hydroxysteroid dehydrogenase type 2 enzyme
5-HT	5-hydroxy tryptamine
ACTH	Adrenocorticotrophic hormone
ANOVA	Analysis of variance
ATL	Adipose triglyceride lipase
AVP	Arginine vasopressin
BW	Body weight
cDNA	Complementary DNA
CORT	Corticosterone
CRH	Corticotrophin releasing hormone
DBB	Diagonal band of broca
DIO	Diet-induced obese rats
DIO-AS	Diet-induced obese acute stress offspring
DIO-CS	Diet-induced obese chronic stress offspring
DIO-NS	Diet-induced obese non-stressed offspring
DoHAD	Developmental origins of health and diseases
DR	Dietary resistant rats
DR-AS	Dietary resistant acute stress offspring
DR-CS	Dietary resistant chronic stress offspring

DR-NS	Dietary resistant non-stressed offspring
ELISA	Enzyme linked immune sorbent assay
FCL	Fresh corpus luteum
FFA	Free fatty acids
GC	Glucocorticoids
GF	Graafian follicle
GnRH	Gonadotrophin releasing hormone
GR	Glucocorticoid receptor
HF	High fat diet
HPLC	High performance liquid chromatography
HSL	Hormone sensitive lipase
LBW	Low birth weight
LH	Luteinizing hormone
LPL	Lipoprotein lipase
ME	Median eminence
MPA	Medial preoptic area
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogenase
NE	Norepinephrine
OCL	Old corpus luteum
PEPCK	Phosphoenol pyruvate carboxy kinase

PVN	Paraventricular nucleus
RIA	Radioimmunoassay
RT-PCR	Real-Time PCR
SCN	Suprachiasmatic nucleus
VAT	Visceral adipose tissue

CHAPTER 1

INTRODUCTION

1. Statement of Purpose

The rapid rise in the incidence of obesity over the past decade demands alternate explanations other than just genetic changes. Lately, much attention has been diverted towards the contribution of early life disturbances to this current obesity epidemic. Several epidemiological studies suggest that nutritional deficiencies or excess during pregnancy can lead to increased susceptibility to develop metabolic diseases in the offspring. Research over the years has added several other potential prenatal factors like stress, alcohol, endocrine disruptors etc which could program the offspring for metabolic diseases in their adulthood [1]. Glucocorticoids are considered as an important hormonal mediator in prenatal programming of diseases. However, the exact mechanism underlying fetal programming is not understood. Hence, we used prenatal stress as a model to study the role of prenatal glucocorticoids in fetal programming of obesity. In order to address the influence of pre-existing genetic predisposition to obesity on the effects of prenatal stress, we used a diet-induced obese (DIO) and dietary resistant rat model for our experiments.

The overall aim of this dissertation is to investigate the differences in the metabolic programming effects of prenatal stress and postnatal HF diet exposure in the DIO and DR rats and also elucidate the mechanisms behind this phenomenon. The studies in the following chapters will first characterize the metabolic phenotype induced by prenatal stress in the DIO and DR offspring. We will then explore if there is association between stress axis activity and the metabolic phenotype observed in the prenatally stressed offspring. The final chapter will study the role of 11β-hydroxysteroid dehydrogenase type 1 enzyme (11βHSD1) in the metabolically active tissues like liver

and adipose tissue in fetal programming of obesity in DIO and DR rats. The current research will provide greater understanding of the mechanisms behind fetal origins of obesity.

2. Obesity Epidemic – A global challenge

The prevalence of obesity is increasing at an alarming rate throughout the world and it is the fifth major risk factor for mortality. Obesity and overweight are believed to be the main causative factors in 44% of type II diabetes (T2D), 23% of ischemic heart disease and 7-41% of cancer in human patients [2]. Obesity is defined as a condition where the body mass index (BMI) is more than 30 kg/m^2. BMI is a number that is calculated using a person's height and weight. It provides a reliable indication of the amount of body fat for most people except for athletes in training. It is commonly used as a screening tool to assess health risk and is one of the best methods for determining obesity and overweight levels in a population [3].

Around the world, 300 million people are estimated to be either obese or overweight. In the Unites States alone, according to a recent statistics from the National Health and Nutrition Examination Survey (NHANES), around 35% of adults and 29% of the women in the reproductive age (20-39yrs) are obese with a BMI >30 [4, 5]. Apart from increasing the complications of pregnancy, obese mothers can directly pass the trait to their offspring [6]. Therefore, it is not surprising that there is an alarming increase in childhood obesity paralleling adult obesity [7]. Corresponding to the prevalence of obesity among adults, the prevalence of childhood obesity has almost tripled in the past 3 decades. In the 2009-10 NHANES survey, 16.9% of children and adolescents were estimated to be obese [8]. Apart from promoting obesity in adult life,

childhood obesity also increases the risk for fractures, breathing problems, hypertension, early insulin resistance and psychological disturbances during childhood [9].

The roots for the onset of obesity are complex. In the modern world, apart from genotype, easy availability of low cost, high calorie fast foods, low physical activity and other socio-economic factors are believed to be the culprits responsible for this obesity epidemic. Although people with high BMI more often have abnormal fat accumulation, it is important to understand that abdominal obesity is an independent risk factor for cardiovascular diseases (CVD) and T2D. This is particularly true with the Asian population, where people have normal BMI but abdominal obesity puts them at higher risk to all diseases similar to obese people. Several organizations like the CDC, the World Health organization (WHO) and International Federation of Sports Medicine, recommend physical activity (minimum of 30 minutes daily) as an intervention for obesity associated health issues. Although it seems like a simple solution, it requires will power and self-discipline in individual patients and the difficulty in attaining this goal might partly explain the continuing rise in obesity rates. Alternatively, understanding the mechanisms behind the development of obesity will help identify therapeutic targets for the treatment of obesity and also prevent its associated cardiovascular morbidity. Along these lines, researchers in the last decade have focused on understanding the developmental mechanisms behind the onset of obesity. It is now an accepted theory that adult diseases like obesity could be "programmed" *in utero.* Several studies have proposed that nutritional status, stress, existing metabolic conditions like diabetes during pregnancy play a key role in the "developmental programming" of obesity. For

my thesis, I am going to focus on the role of elevated glucocorticoid levels induced by prenatal stress as a programming factor in the development of obesity. I will perform my experiments using the diet-induced obese (DIO) model that is an established model of human obesity and its lean counterpart, the dietary resistant (DR) rat.

3. Fetal programming of adult diseases

Genotype, diet and environment or life style factors are known to be associated with the development of obesity and metabolic syndrome in adults. Metabolic syndrome is a complex collection of symptoms or risk factors that predispose an individual to cardiovascular and metabolic diseases. Basically, it includes central or abdominal obesity (characterized by a BMI of >30, or a waist to hip ratio of >0.9 for men, or >0.85 for women), insulin resistance (measured by a fasting blood glucose level of >100mg/dl), dyslipidemia (marked by elevated triglycerides and/or cholesterol levels) and elevated blood pressure that may or may not be managed by medication [10]. An individual who is at risk for obesity, is also at risk for developing metabolic syndrome and its associated disorders. Therefore it is important to understand how obesity develops in order to prevent the transition to metabolic syndrome.

Recent studies have suggested that adverse *in utero* conditions prevailing during pregnancy can induce permanent alterations during development, “program” the fetus and predispose the offspring for adult diseases. Several epidemiological, human and animal studies supports this "developmental origin of health and disease" (DOHaD) concept [11, 12]. Originally termed as the ‘Barker’s hypothesis’, this concept originated from human studies demonstrating that under nutrition during pregnancy results in poor adaptive responses to postnatal challenges like over feeding and stress, resulting in the

development of obesity and the metabolic syndrome. Barker and his colleagues reported increased incidence of cardiovascular diseases and diabetes in men born to moms who received poor nutrition during the Dutch famine in 1944-45 [13].

It is believed that the sub-optimal in utero environment allows for adaptational changes in the fetus that helps in short term survival during gestation. In the post natal period, these adaptational changes would favor diversion of most of the available energy to storage depots like fat in anticipation of adverse nutritional conditions in the future. This phenomenon is termed as the 'thrifty genotype or thrifty phenotype hypothesis'. In contrast to what is expected, if there is postnatal overnutrition, the adaptations developed to survive nutritional scarcity become inappropriate and results in obesity. In simple words, a nutritional imbalance between prenatal and postnatal periods (prenatal undernutrition vs postnatal overnutrition) underlies the predisposition for the development of obesity and metabolic syndrome in the offspring.

a. Birth weight and programming

Low birth weight is an important characteristic feature of the offspring raised in a compromised *in utero* environment. Infants born with low birth weight subsequently undergo rapid catch-up growth in their early life and as a result, are at increased risk for developing obesity, coronary heart disease, non-insulin-dependent diabetes mellitus (NIDDM) and hypertension [14-16]. Several retrospective cohort studies in humans where adult outcomes were correlated with birth weight records, support this relationship between low birth weight and the development of adult chronic diseases [17, 18]. This association is independent of the adult life style factors like social class, smoking, alcohol consumption and lack of exercise. The possibility that genetics could

contribute to these chronic diseases is negated by data from the “twin study”. In this study, only the twin with low birth weight had high blood pressure and developed insulin resistance in adulthood [19]. This observation clearly established the link between low birth weight and cardiovascular and metabolic disease in adulthood. In addition to low birth weight, placental weight and placental-birth weight ratio should also be considered before determining the potential health risk for the offspring. A disproportionately larger placenta leading to higher placental-birth weight ratio has been associated with higher systolic blood pressure [20] and coronary artery disease [21] in the offspring. Moreover, studies indicate that babies whose intra-uterine growth is less compared to their actual growth potential are at risk for developing obesity rather than babies who are just smaller in size [22].

In contrast to undernutrition, maternal overnutrition or gestational diabetes results in increased fetal growth leading to large for gestational age (LGA) or macrosomic babies. Like low birth weight babies, studies have documented that LGA babies are also at increased risk for developing obesity and its associated disorders in adulthood [23]. Hence, it is clear that there is a “U” shaped relationship between birth weight and adult disease risk as studies report that both low and high birth weight babies as compared to normal ones have diabetes in adulthood. Although there is enough evidence to support the association between abnormal birth weight and adult disease outcomes, it is only crude and not a definitive measure for the occurrence of programming *in utero*. This is because there are other studies demonstrating that disturbances like maternal undernutrition during the early gestational period

predisposed the offspring to coronary artery diseases without producing any change in birth weight [24, 25].

Apart from nutritional factors, the realm of prenatal determinants contributing to the fetal programming of adult diseases has expanded over the past 2 decades. Stress, alcohol, smoking and exposure to environmental pollutants with obesogenic properties such as tributyltin, bisphenol and phthalates [1] are some of the other programming factors currently being investigated. In addition to obesity and metabolic syndrome, recent studies have proved that Barker's hypothesis holds true for the increased prevalence of other diseases like allergies, osteoporosis, psychiatric disorders, cancer and coagulation disorders [26, 27]. All these lines of evidence confirm that early life disturbances can produce long lasting effects in the offspring and this leaves open the possibility that prenatal factors in addition to the genotype and environment may play a significant role in the current obesity epidemic.

b. Mechanisms of programming

Though Barker's hypothesis helped us to conceptually understand the developmental origin of diseases, it is still not clear as to the mechanisms by which the fetus is programmed *in utero*. Several mechanisms have been proposed so far to explain the postnatal effects in the programmed offspring, although these are not mutually exclusive. These are described below:

1. Alteration in the ontogeny of the organs (development and morphology)

With respect to organ development, reduced nephron number [28], reduced beta and alpha cell mass in the pancreas [29], increase in red cell mass [30] and long term

decrease in cell turnover in the hippocampus, hypothalamus and pituitary [31] have been reported in the offspring that have been compromised *in utero.*

2. Dysfunction of endocrine systems and metabolic imbalance

Exposure to prenatal restraint stress and undernutrition causes hyperactivation of hypothalamo-pituitary adrenal (HPA) axis resulting in chronic increase in circulating glucocorticoids in the offspring [32-35]. Furthermore, these offspring are hyperresponsive to postnatal stress with increase in circulating adrenocorticotrophin (ACTH) and corticosterone levels and increased expression of hypothalamic corticotropin releasing hormone (CRH) and hippocampal 11β-hydroxysteroid dehydrogenase-1 (11β-HSD1) [36]. Young adults born to moms who experienced stressful events during pregnancy have been reported to be insulin resistant with elevated 2-hour insulin and C-peptide levels [37]. Gestational exposure to high fat diet results in increased expression of metabolic genes such as insulin like growth factor and PPARα in the liver [38]. All the above mentioned changes in the endocrine system have been implicated in the development of T2D, obesity and behavioral disorders.

3. Epigenetics

Most of the studies in the field of fetal programming have been studied in the first generation offspring (F1). But, these *in utero* programming effects are not only restricted to the immediate generation but can extend to subsequent generations as well. Studies have reported transgenerational inheritance with transmission of the developmental effects to the F2 and F3 generations. For example, maternal undernutrition (30% food restriction) in guinea pigs elevated basal cortisol levels and increased interventricular septal wall thickness and left ventricular wall thickness in F1

and F2 male offspring [35]. Similarly, low birth weight due to maternal low protein diet was perpetuated in 2 subsequent generations [39]. Also, prenatal exposure to glucocorticoids resulted in glucose intolerance and altered hepatic gluconeogenic enzyme activity in the F2 male offspring [40]. Transgenerational paternal inheritance has also been recently reported [41]. The fact that both maternal and paternal lines were able to transmit the programming effects and that these changes were transgenerational suggest the involvement of epigenetic mechanisms [42, 43].

Epigenetics is the process of modification of gene expression patterns, which are not directly dependent on changes in primary DNA sequences, but on changes to molecules associated with the DNA, like methylation of DNA or post translational modifications of histones [44]. Alterations in the pattern of methylation leads to either repression or transcriptional upregulation of a gene i.e., hypermethylation of cytosine–guanine dinucleotides (CpG) in the promoter region of a gene inhibits gene expression and vice versa [45, 46] . Methylation changes in the genes coding for HPA related molecules like the glucocorticoid receptor (GR) [47], CRH (Corticotrophin releasing hormone), Arginine Vasopressin (AVP) [48] and the glucocorticoid inactivating enzyme, 11-β hydroxysteroid dehydrogenase type 2 (11βHSD2) [49] have been implicated in the sustained hyperactivation of stress axis observed in the programmed offspring. In humans, hypomethylation of the insulin like growth factor gene was reported in individuals who were prenatally exposed to famine when compared to their normal siblings [50]. These individuals were also at higher risk for developing T2D and coronary heart diseases.

4. Glucocorticoids and fetal programming

a. Glucocorticoid as a programming factor

Several lines of evidence demonstrate that maternal glucocorticoids mediate the effects of the programming factors like diet (undernutrition, low protein and high fat), stress and alcohol on the developing fetus. Experimental protein restriction during pregnancy increases circulating cortisol levels in pigs and monkeys [51, 52]. Maternal adrenalectomy abolishes prenatal protein restriction-induced elevation in blood pressure observed in the offspring [53]. Similarly, high fat diet and alcohol exposure during pregnancy increases maternal circulating corticosterone and ACTH in rodents [54, 55]. From these studies, it is clear that elevated maternal glucocorticoids could be associated with a compromised uterine environment. Pregnant women are also directly exposed to glucocorticoids in the form of treatment for accelerating fetal lung development in preterm delivery and also for treating conditions like congenital adrenal hyperplasia [43]. Hence, researchers have been using various prenatal stress animal models over the past few decades to study the mechanisms behind glucocorticoid mediated developmental programming.

b. Effects of elevated glucocorticoids on the fetus

Physiological levels of glucocorticoids are essential for normal fetal growth as it helps in lung maturation and proper brain development. Glucocorticoids also facilitate tissue differentiation and thus prepare the fetus for delivery. Glucocorticoid receptors are expressed in all the fetal tissues including placenta starting mid-gestation and they mediate all the effects of glucocorticoids [56], [57]. GR mutants die shortly after birth indicating the essential role of glucocorticoids in fetal maturation [56]. On the other

hand, excess glucocorticoid exposure especially during late gestation, when there is accelerated growth, results in growth retardation manifested by reduced placental weight and birth weight of the offspring [58] and this effect of glucocorticoids on birth weight has been documented across all species including humans [59-61].

The actions of glucocorticoids on the fetus are in part mediated through the placenta. Though glucocorticoids are lipophilic molecules and can easily pass the placental barrier, their concentration in the fetal compartment is usually less compared to the maternal compartment. The fetus is protected from excess maternal corticosteroids by placental 11βHSD2, an enzyme which converts active glucocorticoids (cortisol in humans and corticosterone in rodents) to its inactive 11-keto metabolites (cortisone and 11-dehydrocorticosterone respectively) [62]. Reduction in the expression or activity of this enzyme will result in exposure of the fetus to high levels of glucocorticoids culminating in fetal growth retardation [43]. In line with this notion, studies have associated low placental 11βHSD2 activity with low birth weight in humans [63]. Also, administration of 11βHSD2 inhibitors during pregnancy reduces birth weight and predisposes the offspring to hypertension and hyperglycemia later in life [53]. Apart from acting directly on the fetus, glucocorticoids could also act on glucocorticoid receptors in the placenta to impact fetal development. Glucocorticoids affect the placental expression of glucose and amino acid transporters, synthesis and inactivation of steroids and thyroid hormones, and reduce the expression of vascular endothelial growth factor all of which are essential for normal fetal growth [43, 58]. Hence, it is clear that excess glucocorticoids due to decrease in placental 11βHSD2 activity results in placental compromise leading to permanent changes in the offspring.

c. Glucocorticoid mediated programming of HPA axis in the offspring

Most of the postnatal effects of glucocorticoid programming are associated with changes in HPA axis function. In animal studies, behavioral disorders associated with prenatal stress or dexamethasone administration are often accompanied by increases in basal and stress-induced increases in corticosterone and ACTH levels. Studies also report permanent changes in the feedback centers like hippocampus and PVN in terms of expression of 11βHSD1, GR and CRH. These changes have the potential to alter the negative feedback regulation of the HPA axis and possibly result in chronic mild excess in circulating glucocorticoids. If this is accompanied by increased 11βHSD1 in the peripheral tissues like liver and adipose tissue, glucocorticoid actions on its target tissues will be amplified. This indicates that activation of HPA or stress axis plays a very important role in the programming of metabolic disorders. However, the mechanism behind stress-induced metabolic programming of diseases like obesity and T2D is poorly understood.

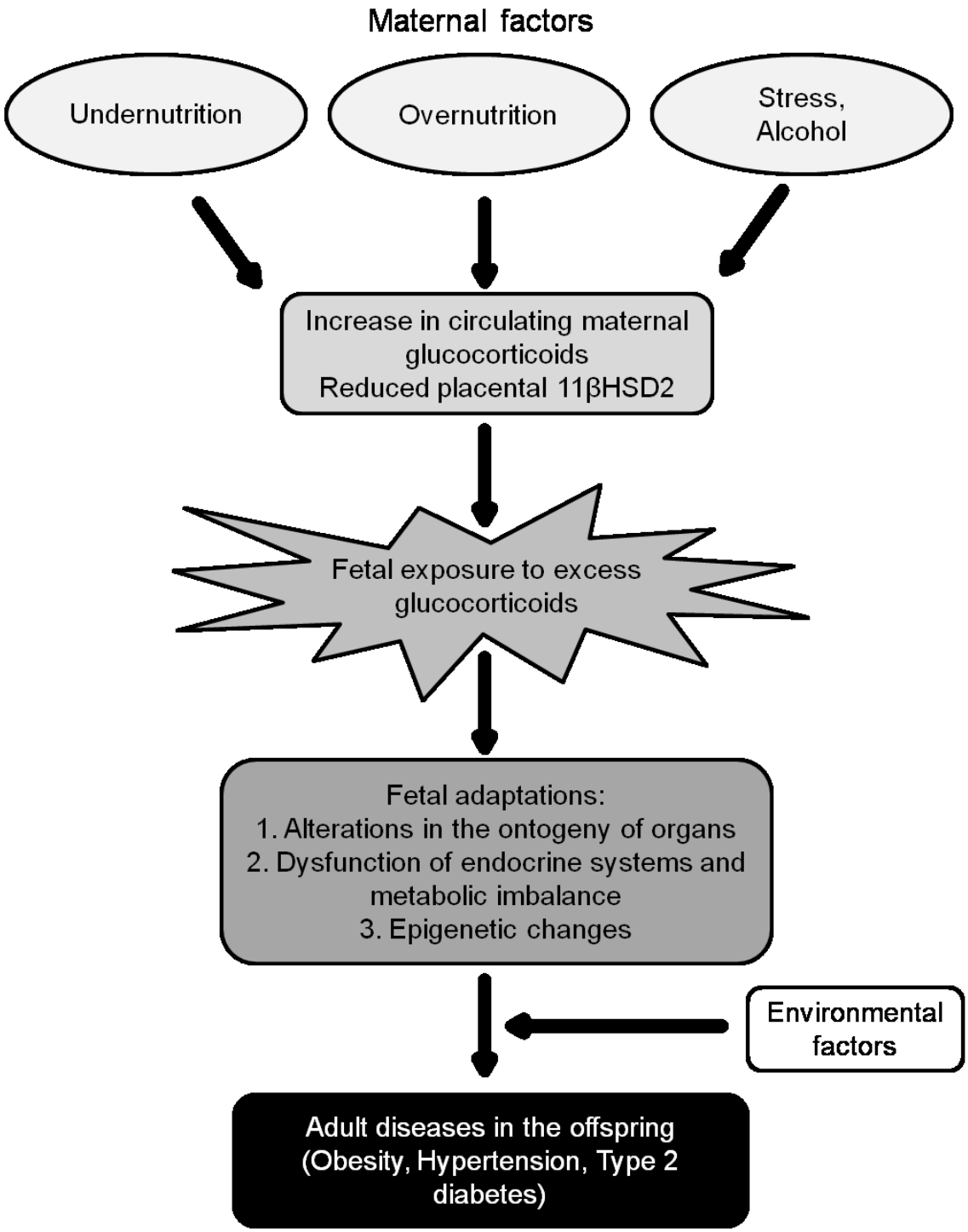


Fig. 1-1: Mechanisms of developmental programming of adult diseases

Adapted from Drake, A.J. et al., Mechanisms underlying the role of glucocorticoids in the early life programming of adult diseases. Clinical Science (2007) **113**, 219–232.

5. Hypothalamo Pituitary Adrenal Axis and Obesity

a. History of the HPA axis-At a glance

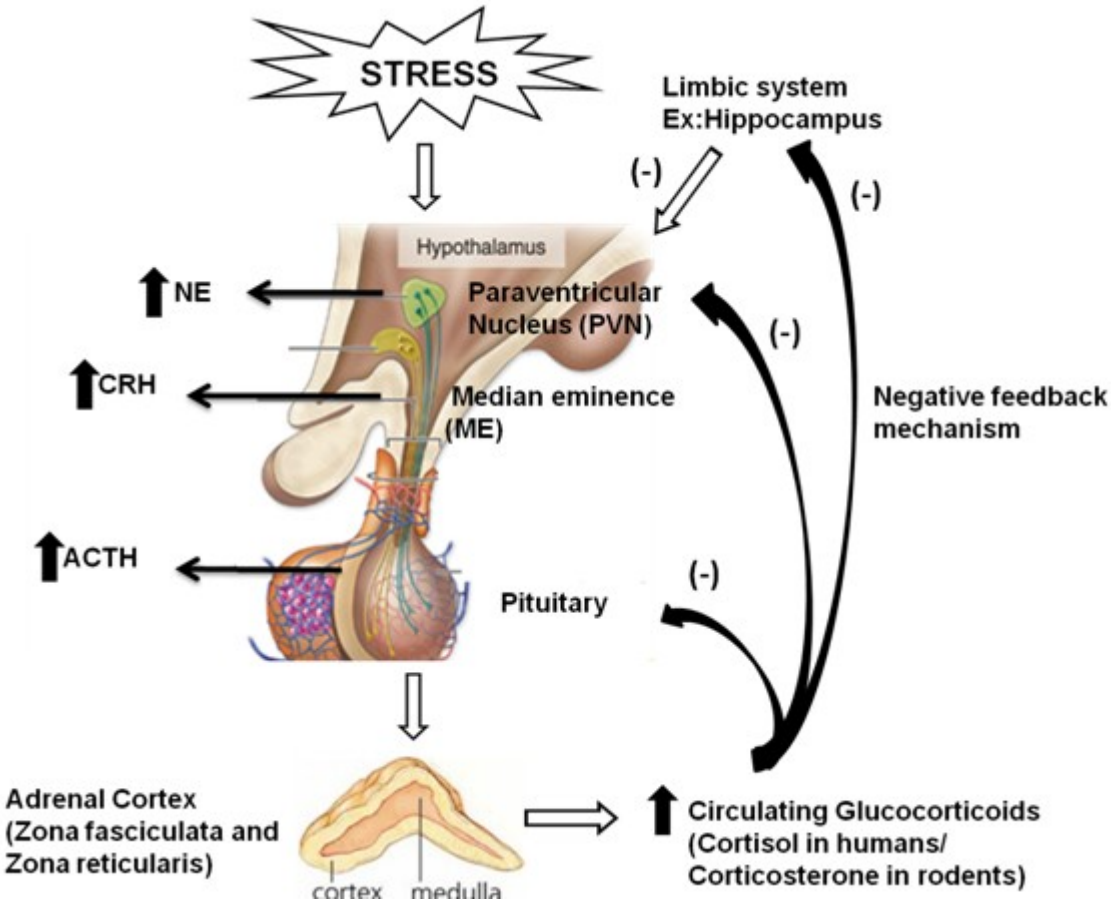
The concept of stress and its physiological significance was first introduced by Walter E. Cannon and Hans Selye. In 1939, Selye's observation of the hypertrophy of the adrenal gland after exposure to a stressor allowed him to postulate that stress activates the adrenal gland [64]. In 1947, John Green and Geoffrey Harris proposed the Neurovascular hypothesis, which states that the 'CNS regulates the activity of the pituitary by means of hormones through the hypophyseal portal system'. Following this, elegant experiments by Saffran et al and Guillemin et al identified Corticotrophin releasing factor (CRH) as the hypothalamic secretagogue for ACTH from the pituitary [65, 66]. In 1950, a rheumatologist, Philip Showalter Hench and biochemists, Edward Calvin Kendall and Tadeus Reichstein shared the Nobel Prize in medicine for their discovery on the clinical benefits of hydrocortisone (Compound E), a hormone isolated from the adrenal cortex, in treating rheumatoid arthritis [67]. Later on, the structure of ovine CRH was characterized as a 41 aminoacid peptide by Vale and colleagues in 1981[68]. In the same time period, arginine vasopressin (AVP) was also identified as a potential ACTH secretagogue [69]. AVP could act independently and also potentiate CRH's effect on ACTH secretion. Apart from acting on the pituitary, both CRH and AVP can directly activate the adrenal cortex to secrete glucocorticoids [70]. Several other factors like cytokines, free fatty acids and triglycerides which increase during conditions like infection and starvation were also found to activate HPA axis. Since its discovery, the viewpoint on glucocorticoids has changed from being a simple anti-inflammatory agent to a deleterious cushingoid agent.

b. Components of the Hypothalamo Pituitary Adrenal Axis

Activation of the stress axis includes a cascade of events culminating in the secretion of glucocorticoids (cortisol in humans, corticosterone in rat/mouse) from the zona fasciculata and zona reticularis layers of the adrenal cortex that help an organism to physiologically adapt and survive a stressful condition. Stress axis is stimulated by activation of the parvocellular area of the paraventricular nucleus (PVN), which houses the major population of corticotrophin releasing hormone (CRH) neurons. PVN receives excitatory inputs through noradrenergic fibers from the brain stem regions like nucleus tractus solitarius (NTS). Upon stimulation by Norepinephrine (NE), PVN neurons secrete corticotrophin releasing hormone (CRH) from their terminals in the median eminence, which then travels through the hypothalamo-hypophyseal portal system and reaches the anterior pituitary. CRH then acts on its receptors located on the corticotrophs in the anterior pituitary to release adrenocorticotropin (ACTH). In addition to CRH, arginine vasopressin (AVP), which is also secreted from the PVN, has also been demonstrated as a powerful ACTH secretagogue [71]. ACTH enters the systemic circulation and reaches the adrenal gland to stimulate corticosterone secretion [72, 73]. Afferent projections to the PVN from other brain regions like noradrenergic brainstem nuclei (A1, A2 and A6) [74], dorsal raphe, the bed nucleus of the stria terminalis (BNST) [75], amygdala and hippocampus [76] are also directly involved in the activation of CRH neurons. Once released, glucocorticoids bind with the glucocorticoid receptors (GR) in the hippocampus, PVN and anterior pituitary and exert a negative feedback effect preventing further CRH or ACTH release, thus terminating stress axis activity (Fig.2) [77].

Fig. 1-2: Overview of Hypothalamo-pituitary adrenal axis

For interpretation of the references to the color in this and all other figures, the reader is referred to the electronic version of this dissertation



c. Negative feedback loop of the HPA axis

Activity of the HPA axis is terminated through a negative feedback loop wherein secreted glucocorticoids act to inhibit its further release from the adrenal gland. Two types of mechanisms exist for glucocorticoid mediated feedback inhibition, namely fast and delayed inhibition [78]. Fast feedback inhibition is dependent on non-genomic mechanisms in the PVN preventing further release of CRH and ACTH and it occurs within minutes. On the other hand, delayed feedback inhibition depends on genomic mechanisms mediated through glucocorticoid (GR) and mineralocorticoid receptors (MR) and takes longer to restrain stress axis activity.

Studies suggest that fast feedback inhibition is mediated through presynaptic retrograde signaling by endocannabinoids synthesized in the CRH neurons upon stimulation of the membrane GR's [79]. Alternatively, glucocorticoids could also act on brainstem noradrenergic neurons to inhibit noradrenergic innervation to the PVN to prevent activation of CRH neurons [78]. With respect to the feedback loop involving limbic structures like hippocampus, amygdala and BNST, the presence of GR's with little or no direct innervations to the PVN suggests the involvement of relay neurons to mediate their effects on CRH release [80]. For example, hippocampus has projections to the brain stem and thus activation of GR's in the hippocampus could directly inhibit catecholaminergic neurons in the brain stem, decrease NE levels in the PVN and terminate stress axis activity. Higher levels of glucocorticoids could also directly act at the level of the pituitary to inhibit ACTH release [81]. Nutrient signals like leptin and free fatty acids are also known to influence stress axis activity. *In vitro* and *in vivo* studies have demonstrated that leptin inhibits corticosterone secretion [82, 83] and this is

mediated through a decrease in NE levels in the PVN [84]. Hence, it is clear that HPA axis is tightly regulated and dysregulation in the feedback control of HPA axis could be deleterious to homeostasis. In fact, alterations in the feedback regulation of the HPA axis has been implicated in neuropsychiatric disorders like Major depressive disorder (MDD) and post-traumatic stress disorder (PTSD) and also in metabolic dysfunction [78].

d. Role of hyperactivation of the HPA axis in obesity

Acute stress results in physiological increases in circulating corticosterone levels which supplies the required metabolic fuel to cope up with a stressful condition. On the other hand, chronic activation of the stress axis might result in excess circulating glucocorticoid levels for a prolonged period of time, which has been implicated in the development of metabolic syndrome [85]. Clinical observations in Cushing's syndrome patients who have elevated glucocorticoid levels, suggested a possible role for excess glucocorticoids in the development of metabolic syndrome. In these patients, there is central obesity and insulin resistance. In a non-human primate model, Shivley and group demonstrated that chronic psychological stress resulted in increased body weight, visceral fat deposition, insulin resistance, glucose tolerance, enhanced cortisol secretion to ACTH stimulation and adrenal hypertrophy [86]. Several clinical studies have also shown an association between urinary-free cortisol levels and higher waist to hip ratio in women and abdominal obesity [87, 88]. Several mechanisms have been proposed to explain how glucocorticoids might promote visceral obesity. Glucocorticoid receptors (GR) are highly expressed in visceral adipose tissue making it more responsive to circulating glucocorticoids. Increased binding of glucocorticoids to GR

results in transcriptional activation of the enzyme, lipoprotein lipase (LPL). This enzyme promotes fatty acid uptake and triglyceride storage, facilitating visceral fat accumulation. This condition is further compounded by the anti-lipolytic actions of insulin during the hyperinsulinemic state [89]. Also, glucocorticoids promote pre-adipocyte differentiation and proliferation. Increase in adipose mass favors a pro-inflammatory state by increasing circulating cytokine levels, which in turn, sustains HPA activation. Taken together, these studies suggest that obesity and stress could reinforce each other and form a vicious circle [90, 91].

6. 11β-HSD1 and regulation of the HPA axis

a. Distribution and physiological functions

The actions of glucocorticoids on target tissues are not only determined by their circulating levels and the availability of receptors, but also on the intracellular pre-receptor metabolism mediated through 11β-HSD enzymes [92]. 11-beta hydroxysteroid dehydrogenase type 1 and 2 (11β-HSD) are enzymes that convert inactive cortisone to active cortisol and vice versa respectively. 11β-HSD1 is a 34kDa protein which acts as a reductase. Its highest expression is seen in the liver but also expressed in lesser quantities in other tissues like adipose tissue, CNS, pancreas [93] and also in the skin [94]. It uses NADPH as a co-factor generated by hexose-6-phosphate dehydrogenase (H6PDH) in the inner lumen of the endoplasmic reticulum (ER) [95]. In contrast, 11β-HSD2 acts as a dehydrogenase and is expressed in mineralocorticoid sensitive tissues like the distal nephron, colon and placenta to inactivate cortisol and prevent its non-specific actions brought about by binding to mineralocorticoid receptor (MR) [92]. The main substrate for 11β-HSD1 is 11-ketosteroid, produced by 11β-HSD2 in the kidneys.

Active cortisol and 11-ketosteroid circulate in equal concentrations during the day, however 95% of active cortisol is in protein bound form sparing a smaller unbound fraction to enter the cell [96]. On the other hand, its inactive 11-ketosteroid form circulates freely and enters the cell where it gets reactivated by 11β-HSD1. Hence 11β-HSD1 is required for tissue-specific amplification of glucocorticoid's actions.

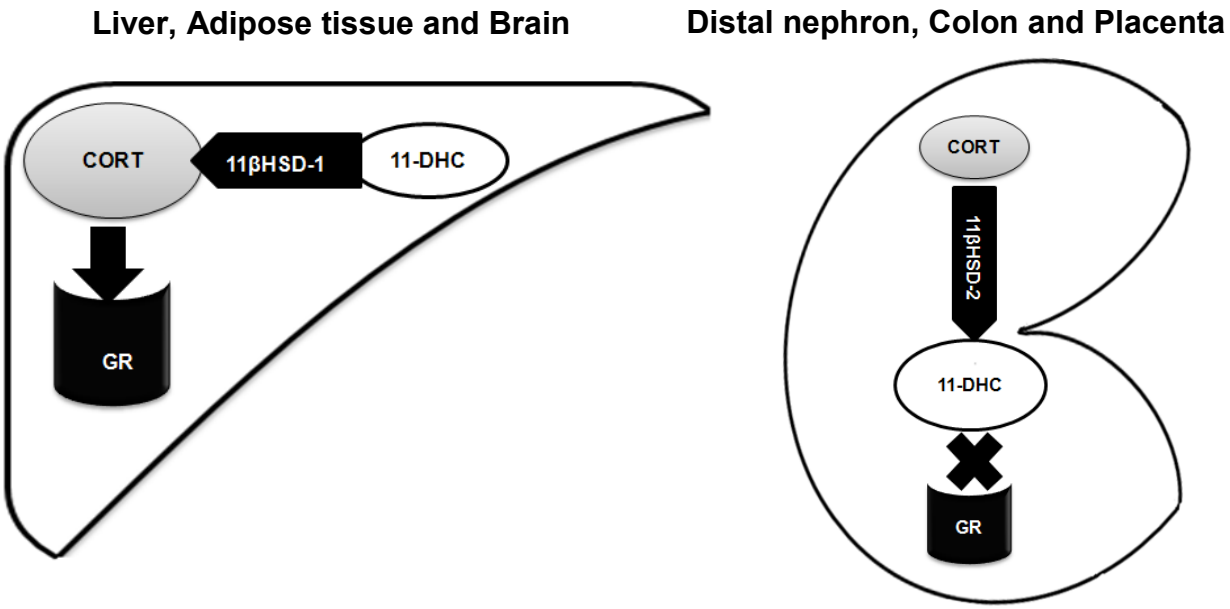


Fig. 1-3: Tissue specific actions of 11βHSD's

11β-HSD1, an enzyme primarily expressed in the liver, adipose tissue and the central nervous system, is involved in the conversion of inactive 11-dehydrocorticosterone (11-DHC) to active CORT (Corticosterone). Circulating CORT has equal affinity for glucocorticoid (GR) and mineralocorticoid receptor (GR). Hence, to prevent the non-specific actions of CORT binding with MR, 11β-HSD2 expressed in the aldosterone sensitive tissues like kidney helps in the inactivation of CORT to 11-DHC, which is then recycled as a substrate for 11β-HSD1.

b. Role of central and peripheral 11-β HSD-1 in obesity

While epidemiological studies demonstrate that higher serum and urinary free cortisol levels are associated with metabolic syndrome [97, 98], studies have also shown normal plasma cortisol levels in obesity [99, 100], which suggests a possibility of altered cortisol metabolism or clearance in these patients. 11βHSD-1, a tissue specific regulator of glucocorticoid action, might be responsible for the altered cortisol metabolism seen in these patients. Irrespective of the circulating corticosterone levels, the intracellular concentration of glucocorticoids available for binding with GR may be increased by 11βHSD-1 to cause the obese phenotype. Supporting this notion, there are numerous clinical studies showing overexpression of 11βHSD-1 in the visceral adipose tissue and liver of obese patients compared to their lean counterparts [98, 101]. Several transgenic animal models have been used to elucidate the role of 11βHSD-1 in glucocorticoid mediated development of obesity. Overexpression of 11βHSD-1, specifically in the adipose tissue of mice, produced features of metabolic syndrome like insulin-resistant diabetes, hyperlipidemia with increased corticosterone content in the adipose tissue [102]. Also targeted deletion of 11βHSD-1 in mice confers resistance to diabetes with attenuated response to high fat diet or stress [103]. Pharmacological inhibition of 11βHSD-1 has also been shown to improve glucose tolerance and slow down the progression of atherosclerosis in a diet induced obese model of metabolic syndrome [104]. All these studies clearly underscore the importance of 11βHSD-1 in metabolic syndrome.

In the brain, 11βHSD-1 is expressed in regions like hippocampus, PVN and cerebral cortex which are all part of the negative feedback loop that helps to decrease

HPA axis activity. 11βHSD-1 is involved in active regeneration of corticosterone inside the neurons, which in turn binds with GR and signals the CRH neurons in the PVN or ACTH secreting corticotrophs in the anterior pituitary to inhibit further secretion of glucocorticoids. Therefore, 11βHSD-1 is thought to play an important role in the inhibition of stress axis activity. Studies with 11βHSD-1 null mice demonstrate elevated basal ACTH and corticosterone levels with hypertrophied adrenals. Also, administration of cortisol prior to restraint stress produced a lesser inhibition of subsequent corticosterone response in 11βHSD-1 null mice compared to WT mice indicating impaired negative feedback in these mice [105]. Hence, impaired negative feedback as a result of reduced expression of 11βHSD-1 in the hippocampus might cause hyperactivation of the HPA axis and result in an obese phenotype.

7. Diet-induced Obese (DIO) and dietary resistant (DR) animal model

Mutations in some of the genes like leptin (ob), leptin receptor (db) and agouti signaling protein (ASP) are associated with the development of obesity [106] and there are transgenic animal models available to investigate these monogenic disorders. However, the cause for the onset of obesity and T2D are multifactorial involving interactions between multiple genes and the environment. Recent studies on whole genome wide analysis in human subjects have demonstrated that at least 18 genes were consistently associated with obesity and T2D [107-110]. Based on these findings, it is clear that in a given obesogenic environment, the individual susceptibility to develop obesity is dependent on a polygenic background [111]. Hence, in order to develop therapeutic targets for obesity and T2D, it is important to perform investigations in a polygenic obese animal model that closely resembles human obesity.

The DIO model is a polygenic obese animal model developed by Levin and group from outbred Sprague-Dawley rats [112]. In westernized society, people in their daily lives consume a high carbohydrate and high energy diet, but weight gain is not uniform across the population. While some are more prone to gain weight, others do not gain weight, even when placed on the same diet. Like humans, Sprague-Dawley rats placed on a high fat diet differ in their capacity to gain weight. The DIO model was developed by feeding adult male and female SD rats with high energy diet (HE diet) for 2 weeks, after which they were classified as DR if they fall in the lowest quartile in weight measurement or as DIO when they fall in the highest quartile. DIO and DR were mated within their phenotype and the offspring were placed back to chow diet. By the fifth generation, inbred DIO gained 90% more weight than DR rats when placed on a HE diet. Even on chow diet, DIO animals gained more weight compared to DR animals. This process continued for over 20 generations for complete phenotypical classification [112]. DIO and DR animal model is a suitable model to study the development of obesity, because they resemble human obesity in their tendency to gain weight on HF diet exposure and they also develop other metabolic alterations similar to obese humans.

Besides their ability to gain weight differentially, the DIO-DR rat model is easy to use for transgenerational studies because their gestation period is short (21 days) and their life span is also relatively short (2-3 years) allowing for close monitoring of growth and feeding habits during the postnatal period. Moreover, stress paradigms during gestation have been previously established in the rat model and the anatomy of the rat brain has been well studied and mapped out.

8. Thesis Objective

The overall objective of my thesis is to understand the metabolic effects of prenatal stress in DIO and DR rats. The studies described in the following chapters were designed to test the following hypotheses: 1) Prenatal stress (acute and chronic stress) predisposes the offspring to obesity and DIO rats will be more susceptible to the adverse metabolic effects of prenatal stress compared to DR rats; 2) Obesity in the prenatally stressed offspring is associated with exaggerated basal HPA axis activity and 3) Prenatal stress increases the expression of 11βHSD1 in the liver and adipose tissue of the offspring resulting in amplification of glucocorticoid's actions. The studies will also help us better understand if pre-existing genetic predisposition to obesity would alter the susceptibility to prenatal factors like stress. Delineating the mechanisms underlying fetal programming of obesity could lead to development of alternate therapeutic options for obesity and metabolic syndrome.

Considering the breeding problems we encountered in the DIO animals, we performed a pilot project (chapter 3) to investigate the reproductive functions in DIO and DR rats, on a regular diet and HF diet. The results from this study suggest that there are inherent reproductive problems in the DIO animals which helped us to design our fetal programming experiments accordingly.

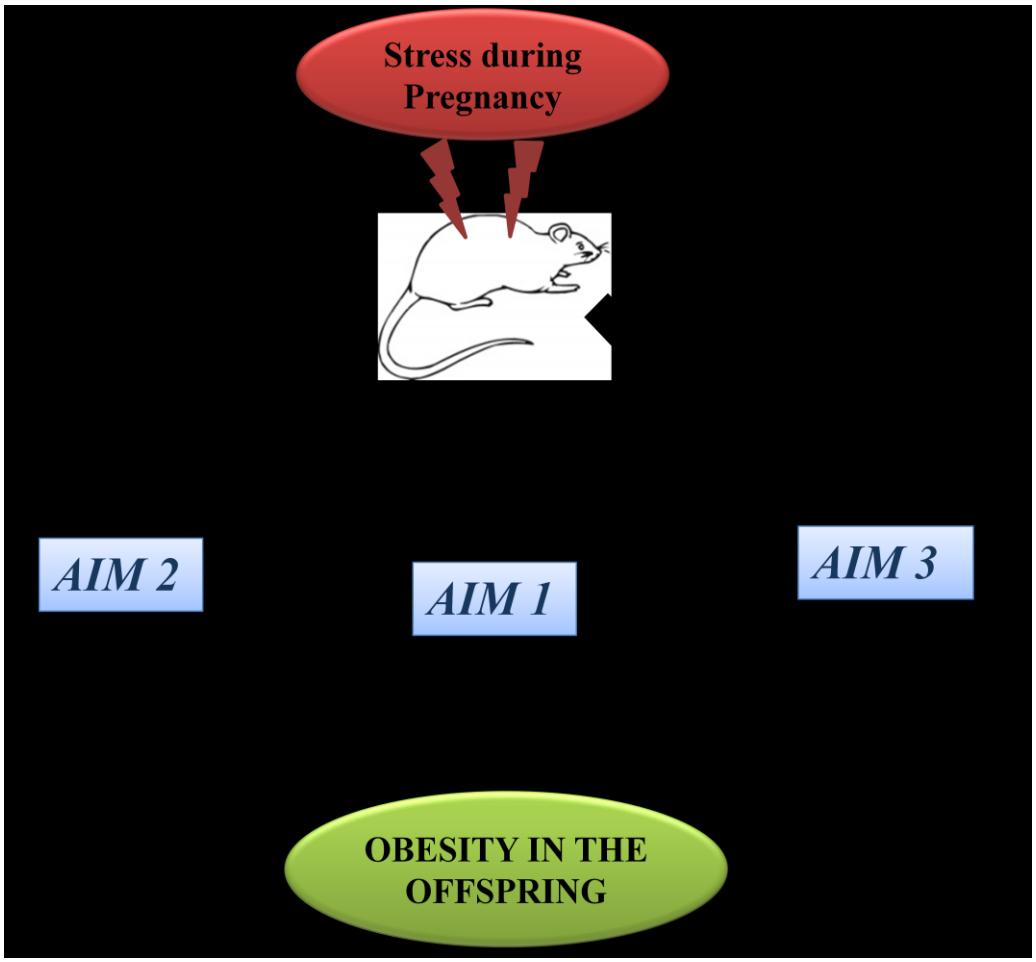


Fig. 1-4 Central hypotheses: Prenatal stress programs the offspring for obesity through hyperactivation of stress axis and tissue-specific programming of 11βHSD1 expression in the liver and adipose tissue.

CHAPTER 2

MATERIALS AND METHODS

1. Animals

DIO and DR breeders were obtained from Charles River laboratories (Wilmington, MA) and were bred in our colony. The F-1 generation offspring from these animals were used in all the experiments. They were housed on a 12:12h light- dark cycle in an air-conditioned room (23±2 °C) with *ad libitum* feed and water. All procedures were in compliance with NIH's guide for the care and use of laboratory animals and were approved by the IACUC at Michigan State University.

2. Treatment

Dietary treatment

DIO and DR animals were weaned at the age of 3 weeks. Animals were fed a regular chow diet after weaning. In the first experiment, in order to test the effects of HF diet on the reproductive axis, 9 week-old DIO and DR female rats were randomly divided into 2 groups each (n=8/group, 4 groups) and placed on a chow diet (23% protein, 72% carbohydrate, and 5% calories as fat with an energy density of 3.11 kcal/g) or HF diet (20% protein, 35% carbohydrate, and 45% calories as fat with an energy density of 4.73 kcal/g; Research Diets, New Brunswick, NJ) for 6 weeks. In the next series of experiments, DIO and DR dams were placed under the acute or chronic stress paradigm as before. Their offspring were placed on regular chow for 6 weeks after weaning and then placed on either chow diet or HF diet for 1 week prior to sacrifice.

Prenatal Stress treatment

Adult DIO and DR females were housed individually with their respective male counterparts. Copulation was confirmed by the presence of sperms in the vaginal smears obtained on the following morning. Animals that were negative for sperms in

their vaginal smears continued to stay with the males. The day when the smears were positive for the presence of sperms is considered as day 1 of gestation. Weekly body weight increments were also used to further confirm the gestational status. Non-pregnant females were eliminated from the study. Pregnant DIO (n=16) and DR animals (n=14) were divided into 3 groups each: Non-stressed (NS) (controls), Acute stressed (AS) and Chronic stressed (CS). Pregnant DIO and DR animals in the non-stressed group were undisturbed throughout the gestational period.

Acute prenatal stress paradigm

DIO and DR animals in the AS group were subjected to the surgical stress of jugular catheterization under isoflurane anesthesia once every week during the 3 weeks of gestation. Isoflurane anesthesia rather than catheterization procedure as such has been shown to produce a 3-fold increase in circulating corticosterone levels compared to baseline levels [113]. One ml of blood was collected from the jugular vein during this procedure. The corticosterone levels achieved from this method is comparable to other prenatal stress methods like restraint stress [114].

Chronic prenatal stress paradigm

Pregnant DIO and DR females were subjected to restraint stress from day 14 to day 21 of gestation. The animals were restrained in transparent glass cylinders during the light hours of the day for 45 minutes, thrice a day. In order to prevent acclimatization to the time of restraint stress, variable time restraint stress protocol was followed [115]. According to this protocol, the time of restraint stress was randomly shifted everyday between certain time periods: 9-11am (morning session), 12-2pm (afternoon session) and 4-6pm (evening session).

Offspring housing conditions

Birth weight of the DIO and DR offspring were recorded within 24hrs of birth. As a measure to avoid litter size and its confounding over or underfeeding variability, litter size was normalized to 8 pups per mom (4 males and 4 females) in all the groups. Male and female offspring from NS, AS and CS groups were weaned onto chow diet at 3 weeks of age. After weaning, the male and female offspring were housed separately in groups of 4 per cage. Weekly body weight and food intake measurements were recorded. At the end of 9 weeks, the offspring from each mom were sub-divided into 2 groups (2 males, 2 females), housed individually and were randomly assigned to chow or HF diet. After 1 week of chow or HF diet treatment, DIO and DR offspring from NS, AS and CS groups were sacrificed by decapitation around noon. Trunk blood was collected at the time of sacrifice. Blood glucose levels were recorded using an Accucheck Aviva glucometer. Serum was separated from trunk blood and stored at -80^{0}C until further analysis. Visceral adipose tissue (VAT) mass and adrenal gland weight was recorded. VAT and liver samples were collected as 2 sets: one flash-frozen and the other formalin-fixed. Brain was collected and stored at -80^{0}C until further analysis.

3. Vaginal cytology

Vaginal smears were obtained between 0800 and 0900 hrs and stained with methylene blue solution (0.5% methylene blue and 1.6% potassium oxalate in water). The stage of the estrus cycle was then determined as described previously [116].

4. Jugular catheterization

Female DIO and DR rats were exposed to a HF diet (45% calories from fat, Research Diets, New Brunswick, NJ) for 6 weeks. At the end of the exposure period, they were implanted with a jugular catheter on the day of proestrus. Catheter implantation was performed between 0800-0900 h under isoflurane anesthesia as described previously [117]. Serial hourly blood samples were collected from 1000 hrs to 1900 h. During each collection, 0.4 ml of blood was collected and the catheter was flushed with heparinized saline to maintain patency. Blood was centrifuged at 2000 rpm for 25 minutes; plasma was separated and frozen at -80^{0}C. Red blood cells from each sample were re-suspended in heparinized saline and re-infused into the animal at the following collection. Animals were sacrificed on the next proestrus day between1500-1600 h by rapid decapitation. Their brains were removed quickly and frozen on dry ice and stored at -80^{0}C until sectioning.

5. Brain microdissection

Serial brain sections of 300μm thickness were obtained using a cryostat (Slee Mainz, London) maintained at -20^{0}C and then transferred to a cold stage maintained at the same temperature. The Paraventricular nucleus (PVN), median eminence (ME), medial preoptic area (MPA), suprachiasmatic nucleus (SCN) and diagonal band of Broca (DBB) were microdissected using the Palkovits' microdissection technique [118] as described previously [119] using a 500μm punch. Nuclei were identified with the help of a rat brain atlas [118]. Care was taken to include all subdivisions of each nucleus. Tissues were stored at -80^{0}C until HPLC analysis.

6. HPLC-EC

NE in discrete hypothalamic nuclei was measured using HPLC-EC as described previously [120]. Briefly, the HPLC-EC system consisted of a LC 20-AD pump (Shimadzu, Columbia, MD), and a phase II, 5-µm ODS reverse phase C-18 column (Phenomenex, Torrance, CA), a SIL-20AC autoinjector, a CTO-20AC column oven maintained at 37°C and a LC-4C detector (Bioanalytical Systems, West Lafayette, IN). The mobile phase contained chloroacetic acid (14.5 g/L), octane sulfonic acid (0.3 g/L), EDTA (0.25 g/L) and sodium hydroxide (4.675 g/L). The pH was adjusted to 3.1 and the mobile phase was filtered and degassed. Acetonitrile 17.5 ml and 13 ml of tetrahydrofuran were added to the mobile phase. The flow rate of the mobile phase was 1.8 ml/min. The sensitivity of the detector was 1 nA full scale, and the potential of the working electrode was 0.65 V. Microdissected hypothalamic nuclei were homogenized in 50 µl of 0.1M perchloric acid and 5-10 µl of the homogenate was used for protein estimation. The rest of the homogenate was centrifuged at 5000rpm for 5 minutes and 15 µl of the supernatant was loaded with 15 µl of the internal standard (dihydroxybenzylamine, 0.05 M) in the autoinjector and injected into the HPLC system. Chromatograms were analyzed for NE concentrations using the Class VP software v. 7.2 (Shimadzu, Columbia, MD).

7. Radioimmunoassay (RIA)

Plasma LH levels were measured by radioimmunoassay. For LH measurements, the standards (rLH–RP-3) and antibody (anti rLH-SII) were obtained from Dr A. F. Parlow (NHPP, NIDDK). LH was iodinated by American Radiolabeled Chemicals, Inc (St. Louis, MO, USA) with the help of Dr. Robert Speth. The LH primary antibody (rLH-

SII) dilution used in this assay was 1:140,625 and the assay was performed as described previously [116, 121]. The intra-assay variability was 5.3±2.9%.

For leptin, insulin and C-peptide measurements a double antibody RIA kit was purchased from Millipore, Billerica, MA, and the samples were assayed in duplicate as per the manufacturer's instructions. Corticosterone was measured from the serum and tissue steroid extracts (liver and adipose tissue) using Coat-a count radioimmunoassay kit purchased from Siemens Health care Diagnostics.

8. Enzyme-Linked Immunosorbent Assay (ELISA)

Plasma estradiol levels were assessed using a competitive EIA kit (Cayman Chemicals, Ann Arbor, MI). The samples were assayed in duplicates as per the manufacturer's instructions. The inter- and intra-assay variability for estradiol assay was 3.75±1.87% and 3.9±0.8%, respectively.

CRH levels in the median eminence were measured using an EIA kit (Phoenix Pharmaceuticals, Burlingame, CA). Briefly, tissue punches of the median eminence were homogenized in 125µl of Phosphate buffered saline (PBS) using an ultrasound sonicator (Kontes, Vineland, NJ). From the homogenate, 50µl was used in duplicates for the measurement of CRH using EIA and 10µl was used for protein estimation. The CRH values were expressed as ng/µg of protein.

9. Western blotting

Liver and hippocampal punches were homogenized in lysis buffer (20mM Tris, 150mM Sodium Chloride, 1mM EDTA, 10% Glycerol and 1% Triton-X) with addition of protease inhibitors (0.5µl of 100mM PMSF, 0.1µl of 100mM Sodium Orthovanadate and Aprotinin). The homogenates were centrifuged at 13,000 rpm for 20 minutes at 4^0C.

Protein levels in the supernatant were estimated using Micro BCA protein assay (Pierce Biochemicals, Rockford, IL). Equal amounts of protein (80µg for liver and 60µg for hippocampus) were resolved on 4-20% NuSep Precast gels (NuSep Inc, Bogart, GA) and transferred to a nitrocellulose membrane. The membranes were blocked for 1 hour at room temperature using Odyssey Blocking buffer (Licor Biosciences, Lincoln, NE). The membranes were then incubated with the following primary antibodies:1:500 anti-11βHSD1 rabbit polyclonal IgG (Santa Cruz Biotechnology Inc, Santa Cruz, CA), 1:500 anti-GR rabbit polyclonal IgG (Santa Cruz Biotechnology Inv, Santa Cruz, CA), 1:1000 anti-β tubulin goat polyclonal IgG (for hippocampal tissues) Santa Cruz Biotechnology Inc, Santa Cruz, CA) and 1:1000 anti- β actin goat polyclonal IgG (for liver tissues) (Abcam, Cambridge, MA) diluted in odyssey blocking buffer with 0.1% tween-20 and 1% thiomersal and incubated overnight at 4^0C. The membranes were washed 4 times with PBS+0.1% tween 20 for 5 minutes each. After washing, the membranes were incubated with infra-red dye conjugated secondary antibodies (donkey anti-rabbit IgG (1:15,000) and donkey anti-goat IgG (1:20,000) (Licor Biosciences, Lincoln, NE) for 50 minutes at room temperature. The membranes were washed again with PBS+0.1% tween 20 for 4 times. The immunoreactive bands were visualized in a Laser scanner (Licor Biosciences, Lincoln, NE) and the intensity of the bands was measured using image J software (NIH, Bethesda, MD). The values were normalized to the loading controls (β-actin or β-tubulin) and expressed as a ratio of 11βHSD1/β-actin or tubulin.

10. Ovarian Histology

Ovaries were collected from the animals (n=6-8 per group) at the time of sacrifice and stored in 10% neutral buffered formalin for 3 days and transferred to 70% ethanol

prior to being embedded in paraffin blocks, and sectioned (5μm). One representative section from both the right and left ovaries of each animal was stained with hematoxylin and eosin and used for follicular count. Histological analysis of the ovarian sections was done using NIS elements BR 3.00 optical microscope and laboratory imaging software (Nikon, Melville, NY). The Graafian follicles and corpora lutea were classified according to Kishi et al., 1999 [122] and counted in a double blind manner. Graafian follicles were characterized by the presence of a confluent antral space filled with fluid with a diameter greater than 350 μm. The size of the follicle was determined by averaging 2 measurements: the largest diameter of the follicle and the diameter perpendicular to it. Fresh CL was identified by the presence of hemorrhage between the luteal cells, and the old CL by the presence of the densely packed luteal cells.

11. Adipocyte area measurement

The formalin fixed visceral adipose tissue were sectioned and stained with hematoxylin and eosin for adipocyte area measurements. The adipocyte area measurements were done using NIS elements microscope under 20X magnification. The adipocyte area was measured at 3 random sites in each animal present in the group. The data is represented as the mean adipocyte area (μm^2) per group.

12. Quantitative RT-PCR

Total RNA was extracted from the liver and hippocampus (n=5-8/group) using GenElute Mammalian Total RNA Miniprep kit (Sigma Aldrich, St. Louis, MO, USA). For adipose tissue, RNeasy Lipid Mini kit (Qiagen, Valencia, CA) was used for RNA extraction. The quality of the extracted RNA was determined using a Nanodrop Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the samples with low

quality RNA assessed by 260/280 ratio (<1.8 or >2.1) were excluded from further analysis. Equal quantities of RNA (500ng) from the samples were subjected to reverse transcription for cDNA conversion with High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). PCR reactions were carried out on ABI 7500 Fast real time PCR system (Applied Biosystems, Carlsbad, CA) using SYBR Green master mix (SABiosciences, Frederick, MD). The PCR reaction mixture included 1 µl cDNA, 10.5 µl RNA grade water, 1 µl primers and 12.5 µl SYBR Green master mix. The PCR conditions were the following: holding stage (95^0C for 10 mts) and cycling stage (95^0C for 15 sec, 60^0C for 1 minute and 72^0C for 35 seconds) followed by melt curve to confirm the specificity of the amplified products. The primers for 11βHSD1 (Ref. Seq. No. NM_017080), Glucocorticoid receptor (Ref. Seq. No. NM_012576) and β-actin (Ref. Seq. No. NM_031144) were purchased from SABiosciences, Frederick, MD. The Ct values were normalized to the house keeping gene, β-actin. The fold change was calculated by $2^{\Delta\Delta CT}$ method.

13. Statistical analysis

Changes in weekly body weight, calorie intake and plasma LH levels were analyzed using repeated measures ANOVA followed by post-hoc Fisher's LSD test. Changes in final body weight, serum leptin, insulin, C-peptide, triglycerides, fat mass to BW ratio, plasma estradiol, NE, CRH and CORT levels were analyzed using two-way ANOVA followed by post-hoc Fisher's LSD test. Western blot and PCR data were also analyzed using two-way ANOVA followed by post-hoc Fisher's LSD test. Simple Linear Regression analysis between leptin and sex steroids and the slope comparison

between DR and DIO was done using Graph Pad Prism software. Changes in ovarian histology did not satisfy the homogeneity of variance rule, hence they were analyzed by a non-parametric Kruskal-Wallis test followed by a post hoc Bonferroni-Dunn test. Results were considered to be significant when $p<0.05$.

CHAPTER 3

HIGH FAT DIET AFFECTS REPRODUCTIVE FUNCTIONS IN FEMALE DIET-INDUCED OBESE AND DIETARY RESISTANT RATS

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1. Introduction

Obesity is a growing epidemic in the United States and the rest of the world [123, 124] and impacts all functions of the body, including reproduction. Obesity has a negative influence on male and female fertility [125-130]. In women, ovulatory disorders are more commonly identified as the major cause of infertility [131, 132] and the incidence is more in obese women than in their lean counterparts [133]. Although there is a strong association between obesity and ovulatory disorders [134, 135] the mechanisms by which obesity affects ovulation still remain unclear.

We wanted to see if reproductive functions are altered in an obese animal model, viz. the diet-induced obese (DIO) rat to understand the mechanisms by which anovulation occurs in obesity. DIO and DR rats are polygenic models of obesity developed by Levin et al [112]. They are derived by selective breeding of outbred Sprague-Dawley rats over generations to retain their propensity to gain body weight or resist weight gain when exposed to a high fat (HF) diet. DIO rats exhibit some of the key features of metabolic syndrome when placed on a HF diet, such as increased body weight and adiposity, hyperinsulinemia, decreased glucose tolerance and hyperleptinemia. The polygenic trait, bimodal pattern of weight gain (DIO vs. DR) and its close resemblance to human obesity makes the DIO/DR rat model a suitable model for the present study than other single gene knock out obese animal models like db/db or ob/ob mice. A handful of studies have previously reported altered ovarian structure and reproductive functions in obese animal models [136-138]. Our laboratory also observed breeding problems with the DIO animals used in the experiments. But the

mechanisms underlying obesity-induced loss of reproductive functions remain to be elucidated.

In the present study, we examined the functioning of the hypothalamo-pituitary-gonadal axis (HPG axis) in DIO rats and compared it to its lean counterpart, the DR rat. The HPG axis is made up of gonadotrophin releasing hormone (GnRH) neurons in the hypothalamus, gonadotrophs in the anterior pituitary and the ovary [139]. GnRH neurons are distributed in the medial preoptic area (MPA), suprachiasmatic nucleus (SCN) and diagonal band of Broca (DBB) of the hypothalamus [140, 141] and their terminals are located in the median eminence (ME). These neurons are influenced by a variety of neurotransmitters and neuropeptides [121, 141-147] and hormones such as estradiol and leptin. One of the main stimulatory neurotransmitters, norepinephrine (NE), increases GnRH secretion to release luteinizing hormone (LH) from the anterior pituitary [148]. The characteristic LH surge that occurs on the afternoon of proestrus is critical for ovulation [148]. We hypothesized that obesity may affect NE levels in the hypothalamus and/or LH secretion from the anterior pituitary to cause ovulatory disturbances.

Since the DIO rat gains more weight when placed on a high fat (HF) diet, we hypothesized that HF diet exposure would worsen the impact on reproductive functions. Therefore, we placed DIO and DR rats on a chow or HF diet and examined the effects on NE levels in specific hypothalamic nuclei involved in GnRH regulation, LH, leptin and estradiol levels in the serum and follicular structures in the ovaries. We used a combination of Palkovits' microdissection, HPLC-EC, radioimmunoassay and histological analysis to achieve this.

2. Experimental Design

In this study, adult female DIO and DR rats were divided into 2 groups (n=8/group) each and placed on a chow diet (23% protein, 72% carbohydrate, and 5% calories as fat with an energy density of 3.11 kcal/g) or HF diet (20% protein, 35% carbohydrate, and 45% calories as fat with an energy density of 4.73 kcal/g; Research Diets, New Brunswick, NJ) for 6 weeks. During the 6-week treatment period, estrous cyclicity was monitored by vaginal cytology. After 6 weeks of HF diet exposure, when the animals were in proestrus, they were implanted with a jugular catheter between 0800-0900 h under isoflurane anesthesia and serial hourly blood samples were collected from 1000 hrs to 1900 h for measuring LH levels. The animals were sacrificed at 1600 h on the next proestrus day subsequent to the day of blood collection and their brains were collected, frozen immediately on dry ice and stored at -80^{0}C until sectioning. Trunk blood was also collected, serum separated and stored at -80^{0}C until analyzed for leptin and estradiol levels. Ovaries were fixed in formalin, sectioned and stained with hematoxylin and eosin for histological analysis. Brains were sectioned and specific hypothalamic regions rich in GnRH neurons like MPA, SCN and DBB were microdissected for NE measurements using HPLC. Regression analysis for leptin and estradiol levels was performed using Graph Pad Prism software.

3. RESULTS

Effects of HF diet on body weight and estrous cyclicity in DIO and DR rats

The effect of HF diet on body weight and estrous cyclicity are depicted in Fig 3-1 A and B. Even on the chow diet, DIO animals weighed more compared to DR rats ($p<0.05$). HF feeding for 6 weeks further increased the body weight (mean ± SE; g) in DIO animals but not in DR animals. (Fig. 3-1A). DIO animals that were fed the HF diet gained the most body weight among all the groups (DIO HF: 349.32±17.1g vs. DR Chow: 205.03±2.6g, DIO chow: 271.64±5.5g; DR-HF: 234.5±6.6g; $p<0.05$). We observed a significant genotype ($F=38.41$, $p<0.05$) and diet effect ($F=11.082$, $p<0.05$) in the body weight gain pattern. However, the interaction between genotype and diet was not significant.

Effects of HF diet on estrous cyclicity in DIO and DR rats are shown in Fig. 3-1B. DR animals on chow diet had regular estrous cycles. Feeding a HF diet to DR animals brought the number of regular cyclers down to 80%. In contrast, only 50% of DIO animals on chow diet had regular estrous cycles. Feeding a HF diet decreased this further to 20%.

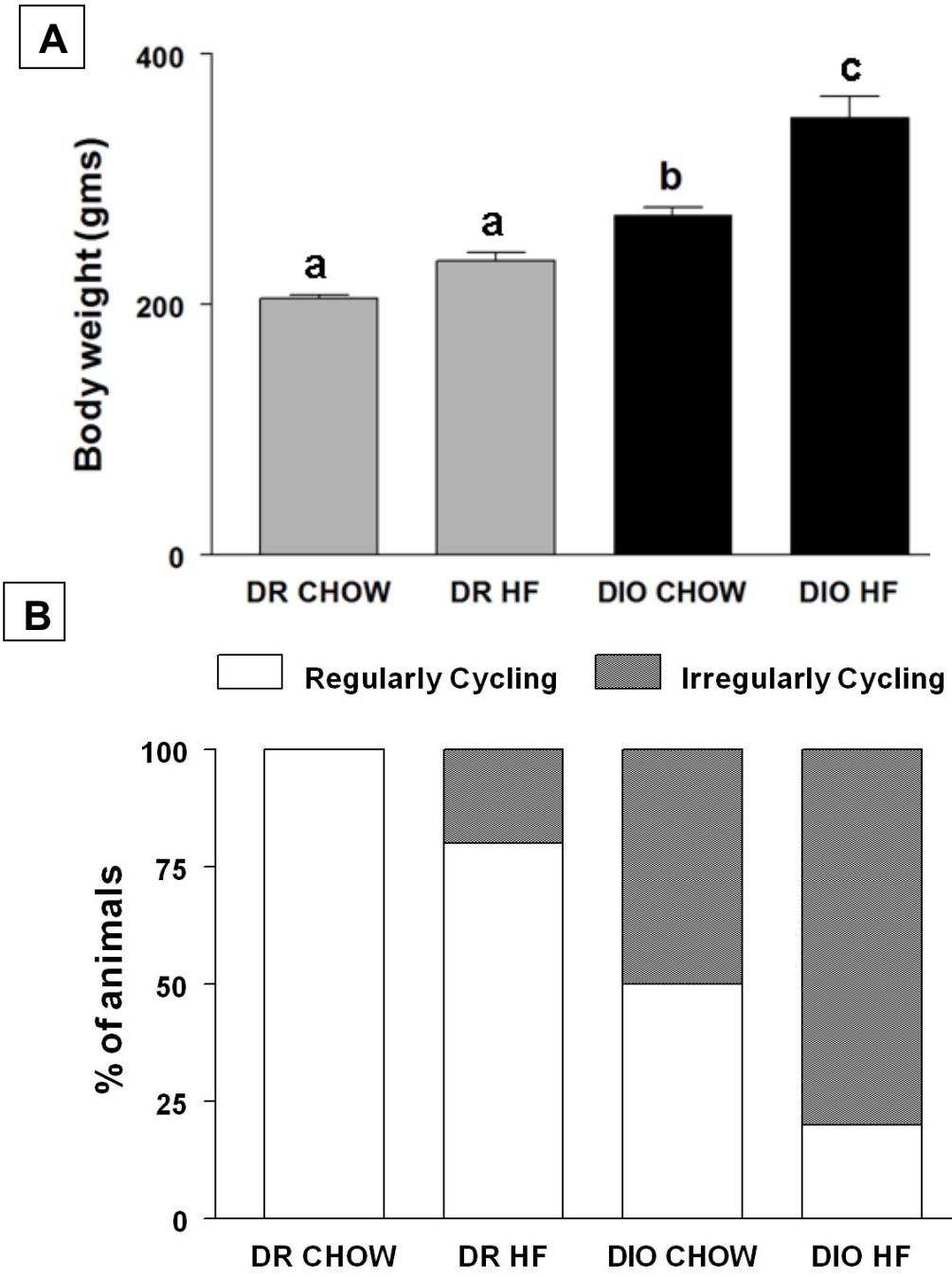

Fig. 3-1 A and B: Effects of HF feeding on body weight and estrous cyclicity

Body weight measurements of DIO/DR female groups (n=8) after 6 weeks of either chow/HF diet are shown in figure 3-1A. Effects of HF diet on estrous cyclicity are shown in figure 3-1B. The white portion of the bar indicates the proportion of the animals that were regularly cycling and the grey portion of the bar indicates the proportion of the animals that were irregularly cycling.

Serum leptin and plasma estradiol levels

Serum leptin and plasma estradiol in the different groups are shown in Fig 3-2 A, B and C. (Fold change in serum leptin levels from DR-chow rats were calculated for all animals in the other three groups and compared statistically using two-way ANOVA. DR-HF animals had a 4 fold increase in serum leptin levels while DIO HF animals had a 8 fold increase in serum leptin levels. Both were significantly different from the DR-chow group). Serum leptin levels (mean ± SE; ng/ml; Fig 3-2A) in DIO rats that were fed chow were 3.19±1.1. Feeding a HF diet increased serum leptin levels by 4-fold in these animals (13.574±2.75; Fig. 3A; $p<0.05$). Serum leptin levels in DR rats fed with chow were 0.758±0.24. Feeding DR animals with HF diet also produced a 4-fold increase in serum leptin levels (4.66±1.2) compared to chow fed rats ($p<0.05$).

Plasma estradiol levels in DIO and DR rats that were treated with chow and HF are shown in Fig.3-2B. Plasma estradiol levels (mean ± SE; pg/ml) in DR rats on chow were 45.2±6.5 and were unaffected by HF diet (33.2±5.0). On the other hand, plasma estradiol levels in DIO rats on chow were 43% less (25.94±4.5) compared to DR rats on chow, although this was not statistically significant. HF diet exposure significantly decreased plasma estradiol levels to 12.73±1.6 in DIO rats compared to the DR group ($p<0.05$). A significant genotype and diet effect was observed with respect to leptin ($F=12.35$ and 19.79 respectively, $p<0.05$) and estradiol levels ($F=15.65$ and 7.86 respectively, $p<0.05$). The interaction between diet and genotype was significant only with respect to leptin levels ($F=4.65$; $p<0.05$).

Regression analysis depicting the association between leptin vs. estradiol in DIO and DR rats are shown in Fig. 3-3C. In DIO rats, there was an inverse relationship between leptin and estradiol that was statistically significant (r^2=0.551, F=8.603, $p<0.05$). No such association was observed in DR animals (r^2=0.004, F=0.054, p=0.819).

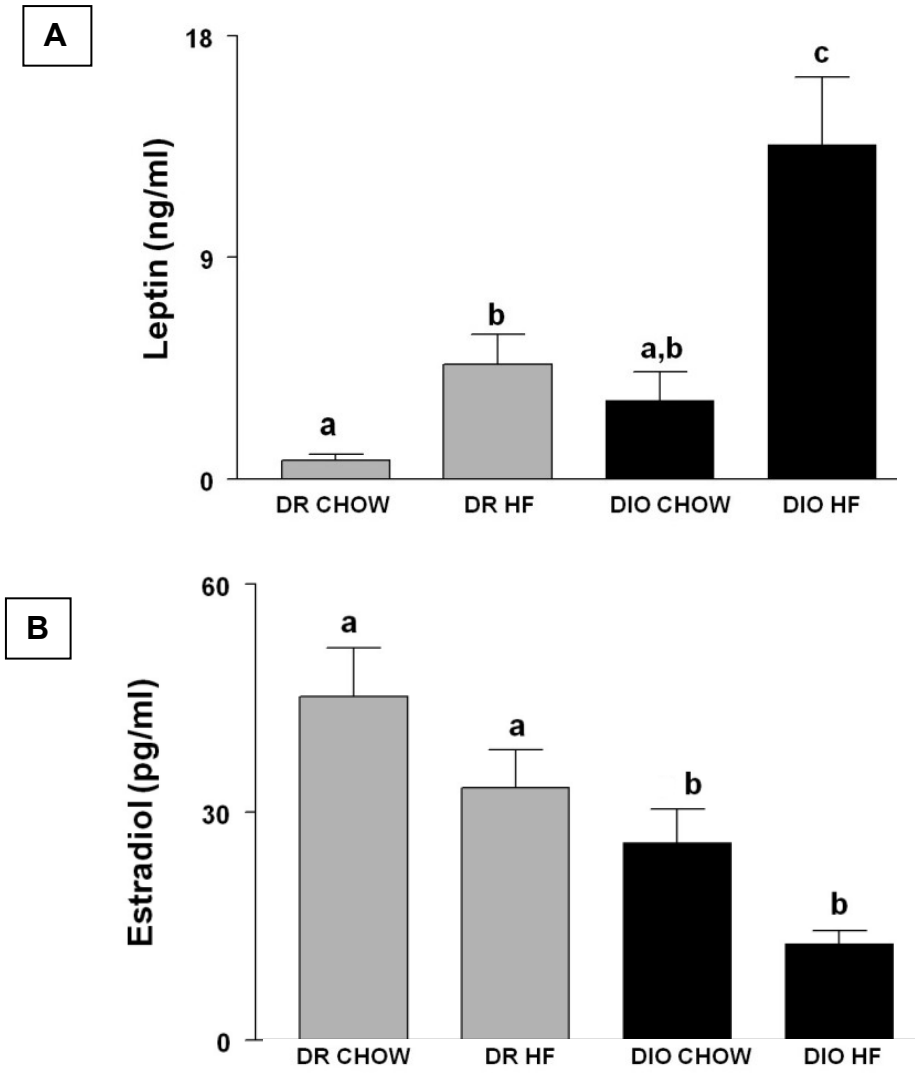

Fig. 3-2 A and B: Effect of high fat feeding on serum leptin and plasma estradiol levels

Serum leptin levels and plasma estradiol in DIO/DR female groups (n=6-8) fed with chow/HF diet for 6 weeks are shown in figure 3-2A and 3-2B, respectively. Groups marked by discrete alphabets are significantly ($p<0.05$) different from each other and the groups indicated with the same alphabet are not statistically different.

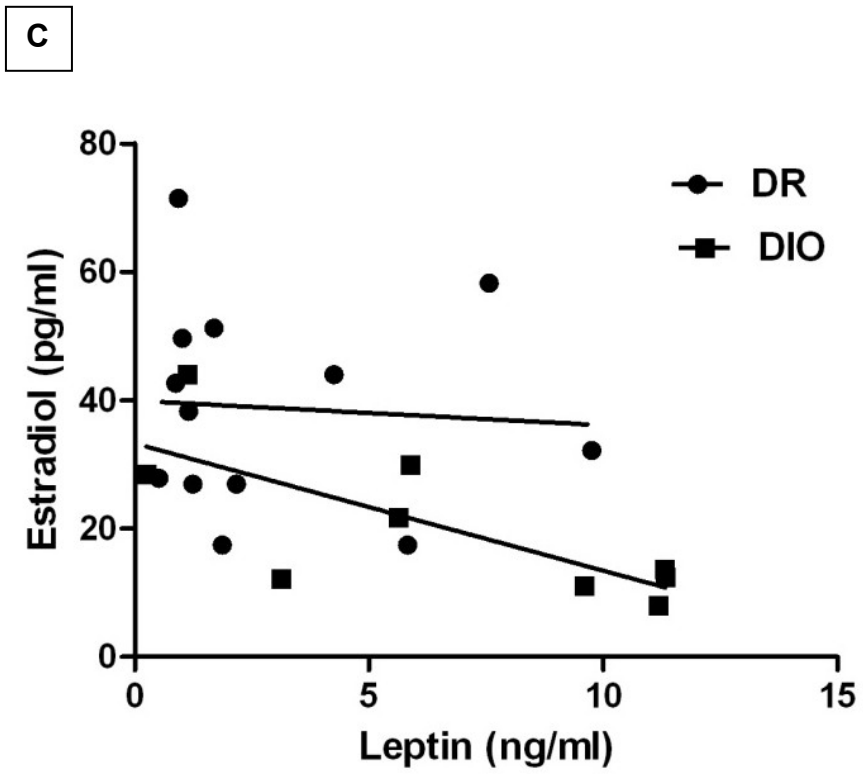

Fig. 3-2 C: Linear regression analysis between serum leptin and plasma estradiol levels

Fig 3-2C provides the linear regression analysis to depict the association between leptin and estradiol. DIO and DR rats (n=5-8 each group) were fed either chow or HF diet for 6 weeks. Relationships are shown in slopes for both DR (line connecting circles) and DIO (line connecting squares) groups. Deviation of the slopes was statistically significant from zero in leptin vs. estradiol (p=0.038) regression in DIO rats, but not in DR rats

NE levels in hypothalamic nuclei

NE concentrations (mean±SE; pg/μg protein) in the MPA, SCN and DBB are shown in Fig 3-3. HF feeding did not affect NE concentrations in the MPA and the DBB in both DIO and DR rats. NE concentrations in the SCN of DIO animals tended to decrease with HF diet but this did not reach statistical significance. No interaction between diet and genotype was observed with NE levels in the hypothalamic nuclei.

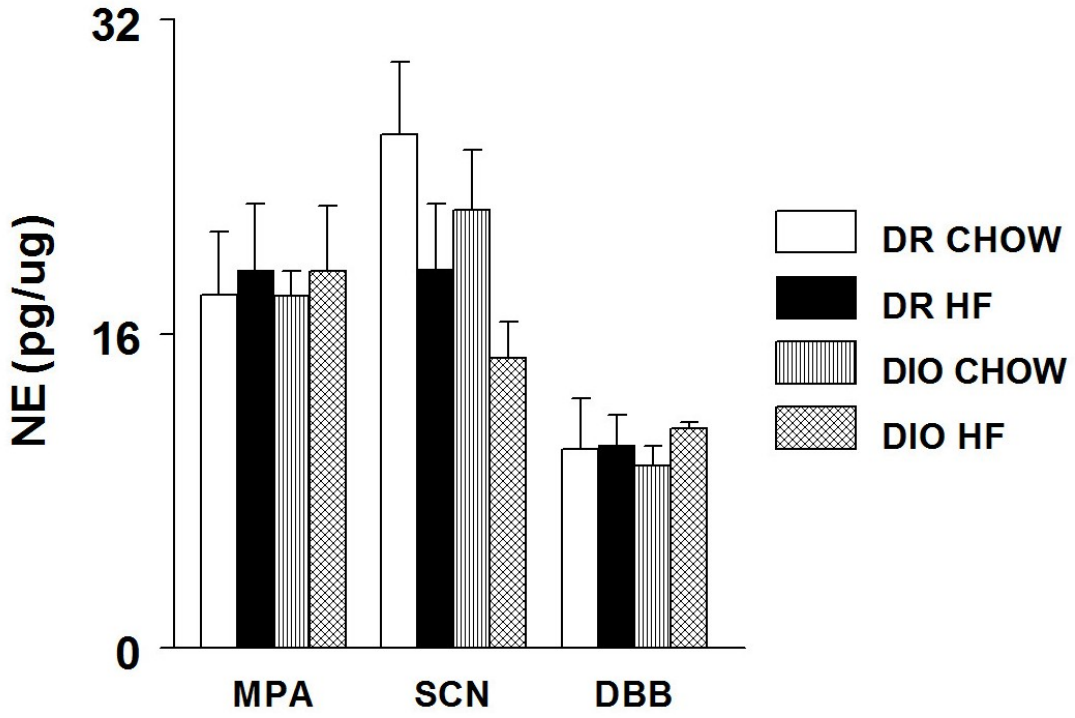

Fig. 3-3: Effects of high fat feeding on NE levels measured in GnRH rich areas of the hypothalamus

Norepinephrine levels (mean±SE; pg/ug of protein) measured by High Performance Liquid Chromatography in MPA, SCN and DBB of DIO and DR female rats (n=5-8) fed with chow or HF diet for 6 weeks are shown. No significant differences were observed among groups in all the 3 regions evaluated for NE levels.

Plasma LH

The LH profile and average plasma LH levels (mean±SE; ng/ml) on the day of proestrus in all the groups are shown in Figs. 3-4 A and B, respectively. In DR animals that were fed chow, LH levels were 1.7±0.412 at 1000 h and increased gradually by 5-fold to 8.4±1.96 ($p<0.05$) at 1600 h and remained around that level until 1900 h. Feeding a HF diet to DR rats suppressed the LH surge. In this group, LH levels were 2.797±0.731 at 1000 h and did not change through the rest of the afternoon. In contrast to DR animals on chow, LH levels in DIO animals on chow were 2.284±0.392 at 1000h, and did not increase to surge levels throughout the afternoon of proestrus. A similar trend was observed when DIO animals were placed on a HF diet. The average LH levels (mean±SE; ng/ml) in the DR chow group on the day of proestrus were 4.498±1.201. Feeding a HF diet significantly reduced average LH levels in DR rats (1.840±.233, $p<0.05$). In contrast to DR animals, the average LH levels were significantly lower in DIO animals whether they were on chow (1.509±.207) or HF (0.935±.120; $p<0.05$). A statistically significant genotype effect ($F=7.4$, $p<0.05$), diet effect ($F= 12.36$, $p<0.05$) and their interaction ($F=8.7$, $p<0.05$) was observed with respect to LH levels on the day of proestrus.

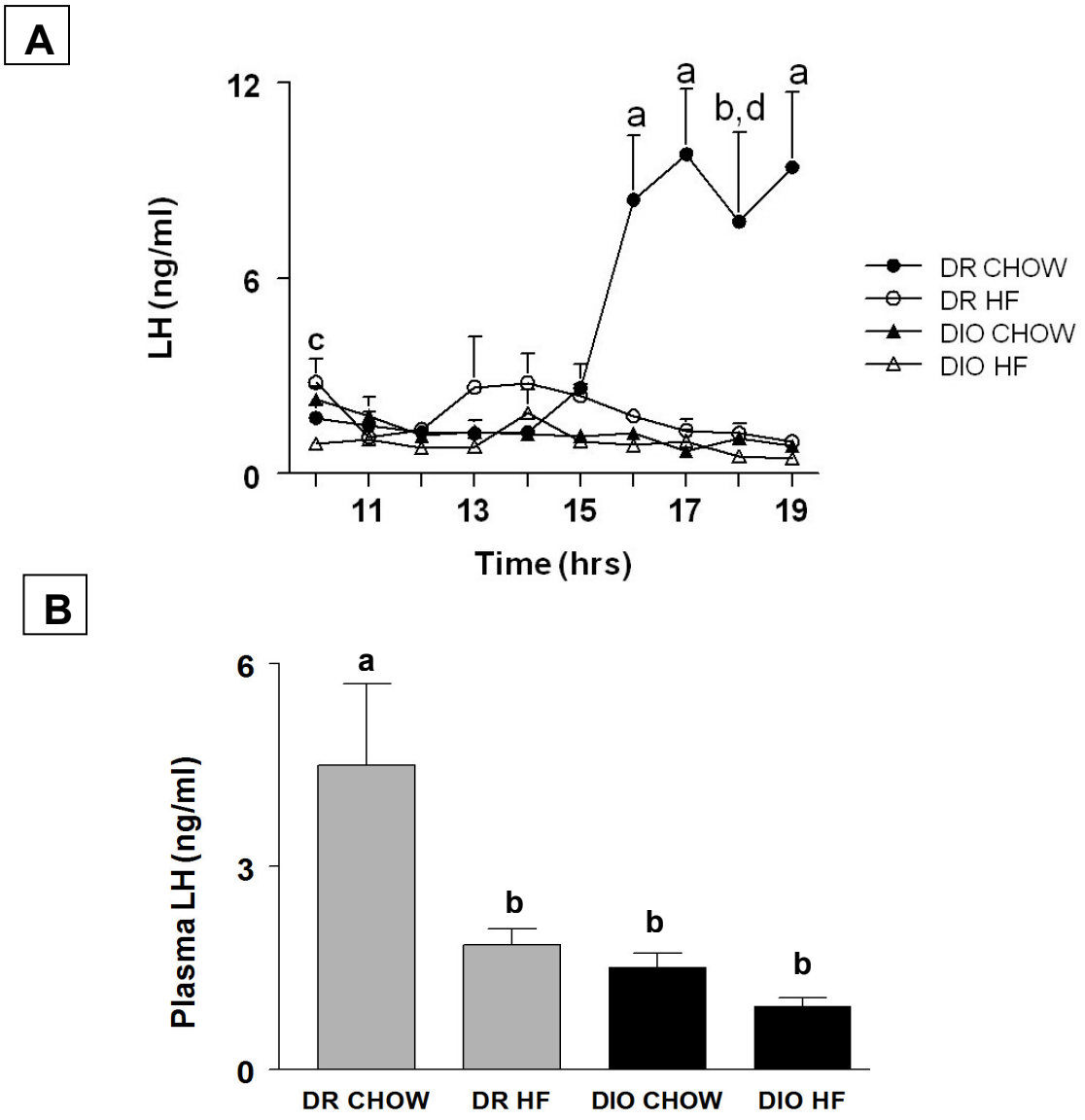

Fig. 3-4 A and B: Effect of HF feeding on LH levels on the day of proestrus in DIO and DR female rats

Plasma LH profile generated by measuring LH levels (mean±SE; ng/ml) at hourly intervals from 1000 to 1900 hrs on the day of proestrus in DIO and DR female rats after 6 weeks of chow/HF feeding are shown in Fig 3-4A. 'a' indicates significant difference ($p<0.05$) from all the other groups, 'b' denotes significant difference ($p<0.05$) between DR CHOW and DIO HF, 'c' denotes significant difference ($p<0.05$) between DR HF and DIO HF groups and 'd' represents significant difference ($p<0.05$) between DR CHOW and DIO CHOW groups. The average levels of LH at different time points throughout the observation period (1000-1900 hrs) on the day of proestrus in DIO and DR female rats after 6 weeks of chow/HF feeding are shown in Fig.3-4B. Groups marked by discrete alphabets are significantly different ($p<0.05$) from each other and the groups indicated with the same alphabet are not statistically different.

Effects of HF diet on the ovary

Representative photo micrographs of ovaries from each of the treatment groups are provided in Fig 3-5A. The numbers of Graafian follicles, old and fresh CLs from all the groups are presented in Fig 3-5B. There were no differences in the number of Graafian follicles between the groups. With respect to CLs, the number of fresh CL was significantly ($p<0.05$) reduced in the DIO groups compared to the DR groups. We also observed a significant decline in the number of old CLs in DIO-HF rats compared to the rest of the groups indicating a further reduction in ovulatory functions. In contrast, HF diet exposure increased the number of old CL's in DR rats ($p<0.05$).

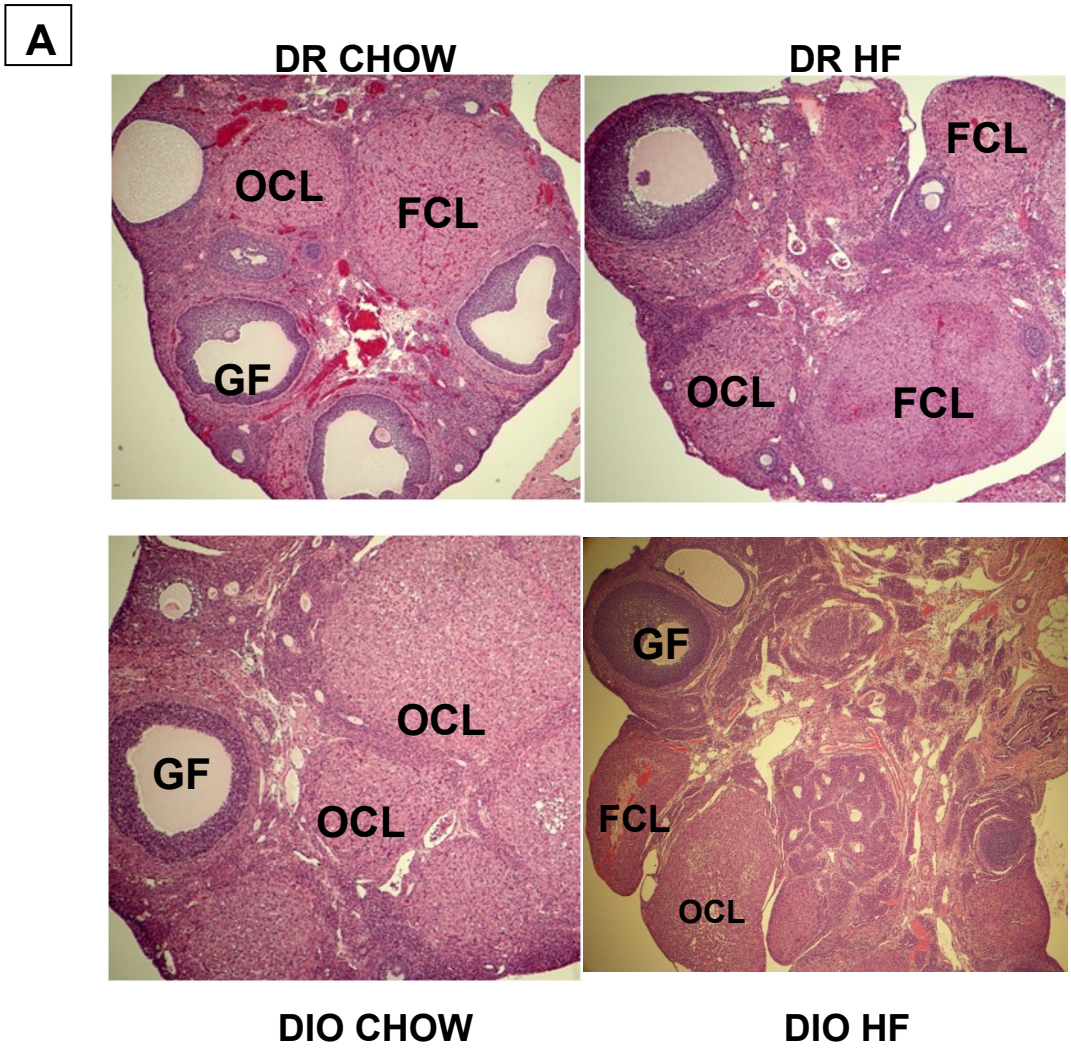

Fig. 3-5 A: Effects of HF feeding on ovaries in DIO and DR rats

Representative histological sections of ovaries (40x magnification) from all the treatment groups (n=6-8 per group) demonstrating Graafian follicles (GF), fresh corpus luteum (FCL) and old corpus luteum (OCL) are shown in Figure 3-5A.

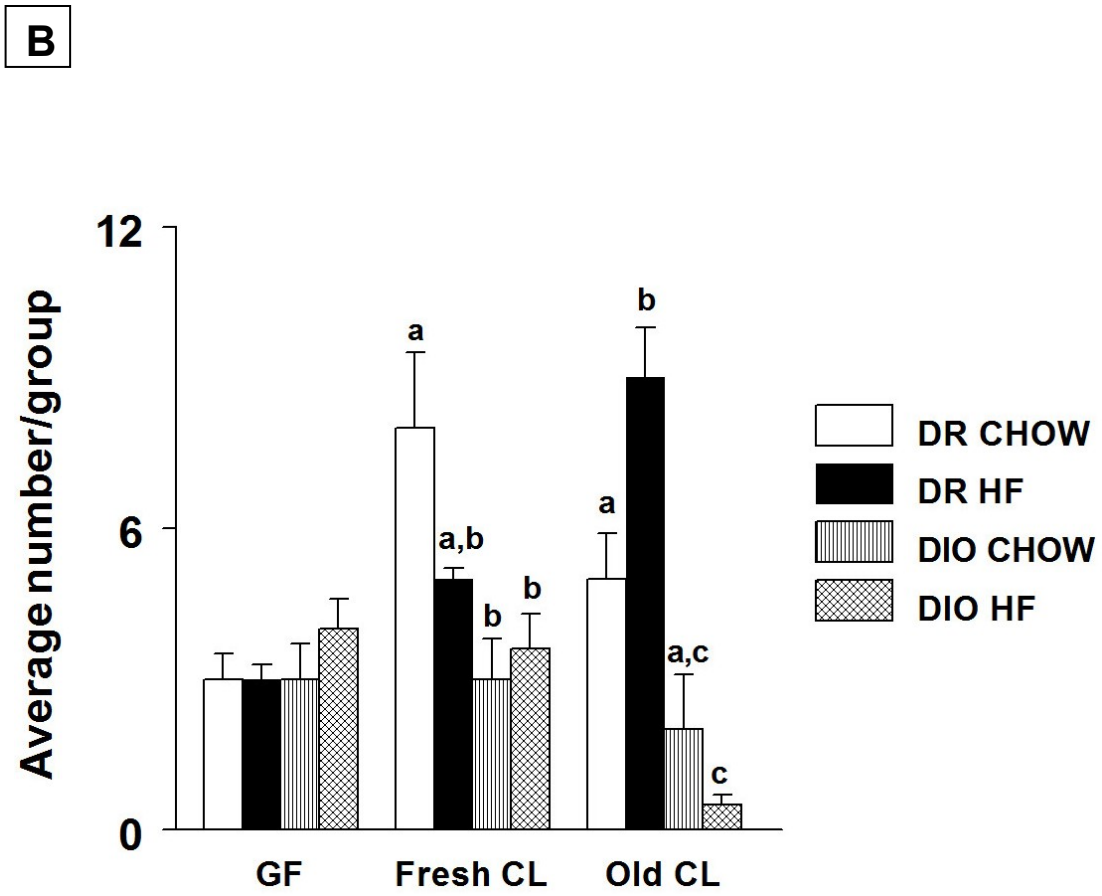

Fig. 3-5 B: Effects of HF feeding on follicular count in the ovaries of DIO and DR rats

Average numbers (mean ± SE) of post-ovulatory fresh corpora lutea (FCL), old corpora lutea (OCL) and Graafian follicles (GF) in the different treatment groups are shown in Fig 3-5B. Groups marked by discrete alphabets are significantly different ($p<0.05$) from each other and the groups indicated with the same alphabet are not statistically different.

4. DISCUSSION

The results from this study provide evidence that female rats that are prone to diet-induced obesity have significant changes in their reproductive axis that may compromise reproductive function. Even on a chow diet, DIO rats have reduced levels of LH resulting in lesser number of fresh CLs indicating impaired ovulation. Feeding them with a HF diet exacerbates this problem leading to a further reduction in the number of fresh and old CLs. The increase in leptin levels, its inverse relationship to estradiol levels and the lack of changes in hypothalamic NE in DIO rats implies that HF diet exposure may affect ovulation by acting through peripheral rather than central mechanisms. Supporting our findings, decreased ovulation and poor breeding performance was recently reported in the New Zealand obese mouse, which is also a polygenic obese model [137]. In an another study, high fat diet (22% fat) exposure for 4 weeks has been shown to cause anovulation with reduced fertilization rate in female mice [138].

There is clear evidence in the literature indicating that obesity impairs reproductive functions [134, 135]. The most commonly observed reproductive disorder in obese women is anovulation [131, 132]. Since ovulation is a centrally regulated phenomenon, we hypothesized that obesity could suppress HPG activity to cause anovulation. However, our findings indicate that NE levels were unchanged in DIO rats compared to DR rats whether they were on chow or HF diet. These findings suggest that obesity and HF diet probably suppress reproductive functions by acting directly on the pituitary or the ovary rather than through central sites.

To test the first possibility, we measured the LH surge on the afternoon of proestrus. While DR rats had a prominent LH surge when they were on a chow diet, placing them on a HF diet produced a marked reduction in surge levels. In contrast to DR rats, DIO animals had low levels of LH even on a chow diet and there was no obvious LH surge. Feeding them a HF diet, did not change the LH profile from the DIO chow group. However, average LH levels appeared to decrease further in the HF fed DIO group than the chow fed group although this was not statistically significant. These results are supported by studies in female rats that have demonstrated that obesity causes a reduction in pulsatile LH release [149] and exposure to a HF diet decreases LH secretion from the pituitary [150]. However, another study has reported an increase in LH levels after HF diet exposure in female rats [151]. The differences in these observations could be due to the duration of exposure to the HF diet and/or the fat content of the diet. Acute exposures appear to increase LH levels [151] while chronic exposures as noted in our study, decreased LH secretion [150].

The reduction in LH levels observed in DIO rats is probably responsible for the low percentage (50%) of animals that have regular estrus cycles even on a chow diet. This indicates that there is a genetic predisposition for reproductive dysfunction in DIO rats. Feeding DIO animals a HF diet further decreased the number of regular cyclers to 20%. A reduction in the number of regular cycles was also observed in DR rats on a HF diet. This suggests that exposure to a HF diet by itself is capable of affecting LH levels and estrus cyclicity. This is supported by another study where estrus cycles were lengthened after HF exposure in rats [152]. The reason for the reduction in LH levels in DIO rats and after HF diet is not clear. Regulation of the preovulatory LH surge is highly

complex involving a number of hormones and neurotransmitters [141, 143, 146]. Of these, the hormone estradiol and the neurotransmitter, NE play a critical role [148]. Since we did not see any change in NE levels in the hypothalamic nuclei that we studied, we measured estradiol levels in the serum. Under normal conditions, circulating estradiol levels increase as ovarian follicles grow in size [153]. It has a positive influence on noradrenergic neurons in the brainstem and also on GnRH neurons in the hypothalamus and luteotrophs in the pituitary [148]. In the present study, circulating estradiol levels were significantly reduced in the DIO-HF group. The reduction in circulating estradiol could have contributed to the reduction in LH levels leading to anovulation in these animals. Also, leptin receptors are expressed in the pituitary and leptin regulates the release of pituitary hormones [154]. Hence, higher leptin levels as seen in obesity could act directly on the pituitary to inhibit LH release. The possibility for the involvement of other neuropeptides like orexins and galanins in reducing LH surge levels also needs to be considered.

The reason for the reduction in estradiol synthesis by the ovary is not clear. Leptin, an adipokine secreted from adipose tissue [155] has been shown to have an important role in reproduction. High levels of leptin are known to impair ovulation and cause infertility [156]. In addition, exogenous administration of leptin reduces ovulation rates both in vivo and in vitro [157]. In the present study, we found that HF feeding in DIO rats significantly increased serum leptin levels and is negatively correlated with serum estradiol levels. There is evidence in the literature to support the view that leptin can interfere with estradiol synthesis in the ovary. Leptin receptor mRNA expression has been reported in the rat ovary and leptin can differentially modulate its own receptor

expression [158]. In vitro studies using isolated human and rat granulosa cells provide evidence that leptin is inhibitory to steroid production [159-162]. The reduction in ovarian steroid synthesis is achieved mainly through the inhibition of pregnenolone synthesis, which is the precursor for estradiol [162]. Results from our study indicate that leptin and estradiol levels in DIO animals are inversely correlated with each other. It is possible that the increase in leptin levels observed in the DIO-HF group can affect estradiol synthesis in the ovaries. Apart from leptin, studies also suggest the involvement of other adipokines like adiponectin in ovarain steroidogenesis. Adiponectin receptors are expressed in the granulosa cells of the ovary (rat, human and chicken) [163-165] and has been shown to stimulate synthesis and secretion of progesterone and estradiol. Hence, it is possible that adiponectin levels are decreased in DIO rats to cause a decline in serum estradiol levels, but this needs further investigation.

The net reduction in LH levels is clearly responsible for the reduced ovulation as indicated by the results from ovarian histology. Although there appeared to be an increase in the number of Graafian follicles in the DIO groups, it was not statistically significant. Another study that involved HF diet, also did not observe a cystic ovarian morphology with HF diet exposure [166].

Overall, the results from the present study indicate that HF diet increases serum leptin levels in DIO animals that are inversely correlated to estradiol levels. The elevation in leptin levels is likely to cause a reduction in estradiol synthesis by a direct action on the ovary. The reduction in estradiol could lead to reduced LH secretion on proestrus and thus result in decreased ovulation. Further mechanistic studies are

needed to prove the causal relationship between leptin, estradiol and LH in the context of obesity. However, the inherent reproductive problems observed in the DIO animals, even on a regular chow diet, prompted us to increase the number of animals used for the experiments described in the following fetal programming studies to obtain enough number of offspring.

CHAPTER 4

DIFFERENTIAL EFFECTS OF PRENATAL STRESS ON METABOLIC PROGRAMMING IN MALE DIO AND DR RATS

1. Introduction

The unrelenting increase in the prevalence of obesity has made it a leading health issue around the globe [2]. Obesity not only affects adults, but recently, there is a disturbing increase in the prevalence of childhood obesity [8]. In addition to the huge economic burden, obesity is an independent risk factor for the development of cardiovascular diseases and type 2 diabetes [9]. So far, genotype and lifestyle factors have been the primary focus of research in the field of obesity. Lately, the mechanisms underlying obesity have been the primary focus of several studies in order to develop a strategy to counter this epidemic. Compromised in utero conditions due to maternal under or over-nutrition, maternal stress, exposure to environmental obesogens etc. can induce permanent alterations in the developing fetus and increase their susceptibility to diseases like obesity and diabetes in the offspring [11, 12]. This concept also called as developmental origins of health and disease (DoHAD) or 'Barker hypothesis' has been widely accepted and expanded to other conditions like allergies and schizophrenia in recent years [167, 168]

Supporting Barker's hypothesis, several studies involving animal models with a compromised *in utero* environment like altered nutritional status (low-protein, calorie restriction) suggest that these factors result in low birth weight in the offspring. Offspring with low birth weight will subsequently undergo catch up growth which puts them at higher risk for developing obesity, hypertension and diabetes in the adulthood [14-16]. Apart from nutritional alterations, maternal stress, which is known to increase circulating glucocorticoids during pregnancy, is also known to program the offspring for metabolic diseases. Numerous studies in rats and primates suggest that prenatal stress or

dexamethasone administration reduces birth weight, increases the susceptibility to postnatal high fat diet exposure resulting in glucose intolerance, insulin resistance, and reduced β cell mass. Stress also alters the expression of lipogenic and gluconeogenic enzymes in adipose tissue and liver independent of changes in body weight [169-171]. In humans, prenatal stress in the form of maternal bereavement or natural disasters during pregnancy has been shown to increase the risk for obesity in the offspring in later childhood [172, 173].

There is ample evidence in the literature to support the metabolic programming effects of prenatal stress both in humans and animal models as discussed previously. However, it is not clear if the susceptibility to the prenatal stress-induced programming is uniform across the population. Previous studies from our lab and others have shown that DIO and DR rats respond differentially to stressors including a dietary stressor i.e., high fat diet [174, 175]. This raises the possibility that obese and lean pregnant women may respond differentially to prenatal stress which in turn could have distinct long term effects in the offspring. In addition, considering the fact that in the United States around 29% of the women in the reproductive age (20-39yrs) are obese [4], it is also important to address if pre-existing genetic predisposition to obesity during pregnancy would alter the responses to prenatal stress in the offspring.

The metabolic effects of prenatal stress vary depending on the timing (early, mid or late gestation) and type of stressor (mild vs severe, repetitive vs varied). Chronic variable stress during mid-late gestation and not during late gestation resulted in long-lasting changes in body weight, leptin and blood glucose levels in the offspring [176]. In humans, exposure to stress after mid-gestation increased the risk for low birth weight in

the new-born [177]. Further, repetitive versus varied stress produced distinct behavioral phenotypes in the offspring [178]. All these studies clearly indicate that the nature and timing of stress play an important role in determining the type of outcomes in the programmed offspring. Hence, in the present study we wanted to test the metabolic effects of acute (surgical stress once a week during 3 weeks of gestation) vs chronic restraint stress (restraint stress during late gestation) in DIO and DR offspring.

We hypothesize that 1) Prenatal stress predisposes the offspring to obesity and DIO rats will be more susceptible to the adverse metabolic effects of prenatal stress compared to DR rats; 2) Postnatal exposure to high fat diet will further exacerbate prenatal stress-induced metabolic alterations and 3) there will be differential phenotypic outcomes to acute vs chronic prenatal stress in DIO and DR rats.

2. Experimental Design

In the present study, pregnant DIO (n=16) and DR (n=14) female rats were randomly divided into 3 groups each: Non-stressed Controls, Acute prenatal Stress and Chronic prenatal Stress group. The animals in the non-stressed group underwent normal gestation and were not disturbed throughout their gestation. The pregnant rats of the acute prenatal stress group underwent surgical stress (jugular catheterization procedure under isoflurane anesthesia) once every week during their gestational period. Also, the chronic prenatal stress group animals were subjected to restraint stress where the animals were immobilized in transparent plastic cylinders with air holes for breathing. The restraint stress procedure was repeated thrice daily during the last week of gestation (day 14-21 of gestation in rats). The chronic stress procedure was carried out randomly within certain time periods (9-11am, 12-2pm and 4-6pm) to prevent habituation of the animals to the stress timing.

The day when the litter was seen in the cage was designated as postnatal day 0 and the birth weight of the offspring were recorded within 24 hours of birth. The litter size was normalized to 8 pups (4 males and 4 females wherever possible) to avoid variability associated with nursing. The offspring were weaned onto a chow diet at 3 weeks of age and housed in groups of 4 according to the sex and treatment groups. Weekly body weight and food intake measurements were recorded. At the end of 9 weeks of age, the animals in each group were sub-divided into 2 groups and were either exposed to HF diet (20% protein, 35% carbohydrate, and 45% calories from fat with an energy density of 4.73 kcal/g; Research Diets, New Brunswick, NJ) or a regular chow diet (23% protein, 72% carbohydrate, and 5% calories from fat with an energy density of

3.11 kcal/g) for 1 week. Finally, there were 6 groups each in DIO and DR phenotypes as listed below:

1. DIO-NS chow	1. DR-NS chow
2. DIO-NS HF	2. DR-NS HF
3. DIO-AS chow	3. DR-AS chow
4. DIO-AS HF	4. DR-AS HF
5. DIO-CS chow	5. DR-CS chow
6. DIO-CS HF	6. DR-CS HF

At the end of the 10^{th} week, all the animals were sacrificed around noon (11 to 2 pm). Blood glucose levels were measured from the trunk blood using a handheld glucose meter. Trunk blood was collected, serum separated and stored at -80 $^{\circ}$C until further analysis. Visceral adipose tissue (VAT) mass (epididymal fat not included) was collected, weighed and expressed as VAT/body weight ratio. Serum was analyzed for leptin, insulin and C-peptide levels using commercial RIA kits (Millipore, Billerica, MA). Serum triglyceride levels were measured using a colorimetric assay (Cayman Chemicals, Ann Arbor, MI).

3. Results

Birth weight in prenatally stressed male DIO and DR offspring

Birth weight (means±SE, g) of the male (n=12-30/group) DIO and DR offspring whose dams underwent normal gestation (non-stressed), acute stress (surgical stress once a week during gestation) or chronic restraint stress (thrice daily from day 14-21 of gestation) are shown in Fig 4-1. Acute prenatal stress (AS) did not have any effect on birth weight in both phenotypes. In contrast, chronic prenatal stress (CS) significantly decreased birth weight in DR offspring (6.18±0.07) when compared with their non-stressed counterparts (NS; 6.65±0.12, $p<0.05$). However in DIOs, chronic stress did not show any effect on birth weight (5.45±.13) compared to non-stressed controls (5.67±0.08). Interestingly, irrespective of the prenatal stress procedure, male DIO offspring had significantly lower birth weight compared to all DR groups.

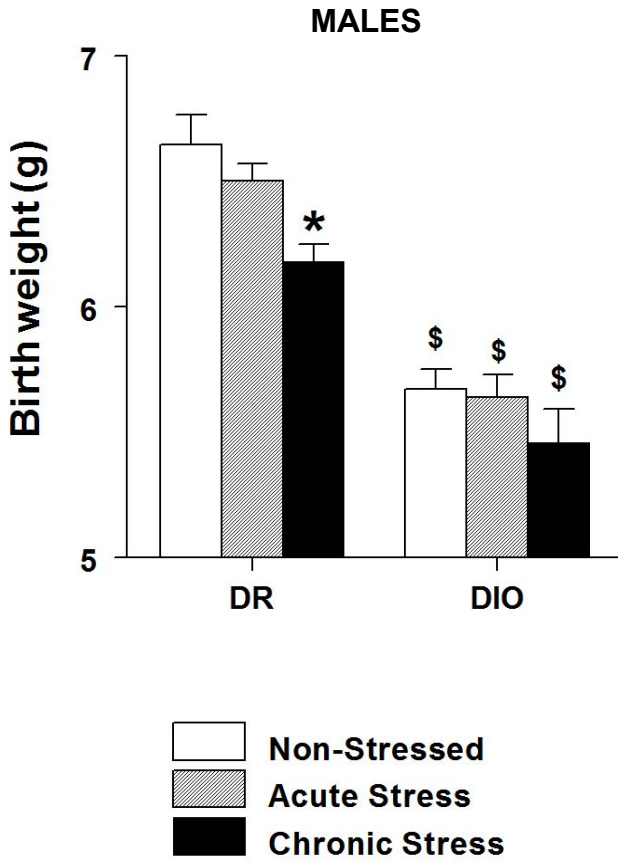

Fig. 4-1: Effects of prenatal stress on the birth weight of male DIO and DR offspring

Birth weight (means±SE, g) of male DIO and DR offspring whose dams underwent normal gestation (non-stressed), acute stress (surgical stress once a week during gestation) or chronic restraint stress (thrice daily from day 14-21 of gestation) are shown in Fig 4-1. '*' $p<0.05$ compared to non-stressed group within genotype. '$' $p<0.05$ compared to all DR groups.

Body weight (BW) until weaning

Body weight (BW, means±SE, g) of the male (n=11-21/group) prenatally stressed DIO and DR offspring are shown in Fig 4-2 A and B respectively. In the first postnatal week, there was a significant reduction in the BW of CS DIO (12.3±0.2) and DR (13±0.3) male offspring compared to their NS counterparts (14.4±.416 and 15.4±0.2 in DIO and DR respectively) (Fig 4-2 A-B) ($p<0.05$). In the DIO group, male offspring subjected to AS weighed significantly more during postnatal week 1 (15.8±0.4 vs 14.4±0.4, $p<0.05$), postnatal week 2 (31.3±0.6 vs 29.42±0.4, $p<0.05$) and postnatal week 3 (49.45±0.9 vs 46.05±0.6, $p<0.05$) when compared to the NS group (Fig. 4-2A). No such differences in the BW were noticed in the AS group of DR male offspring (Fig. 4-2B).

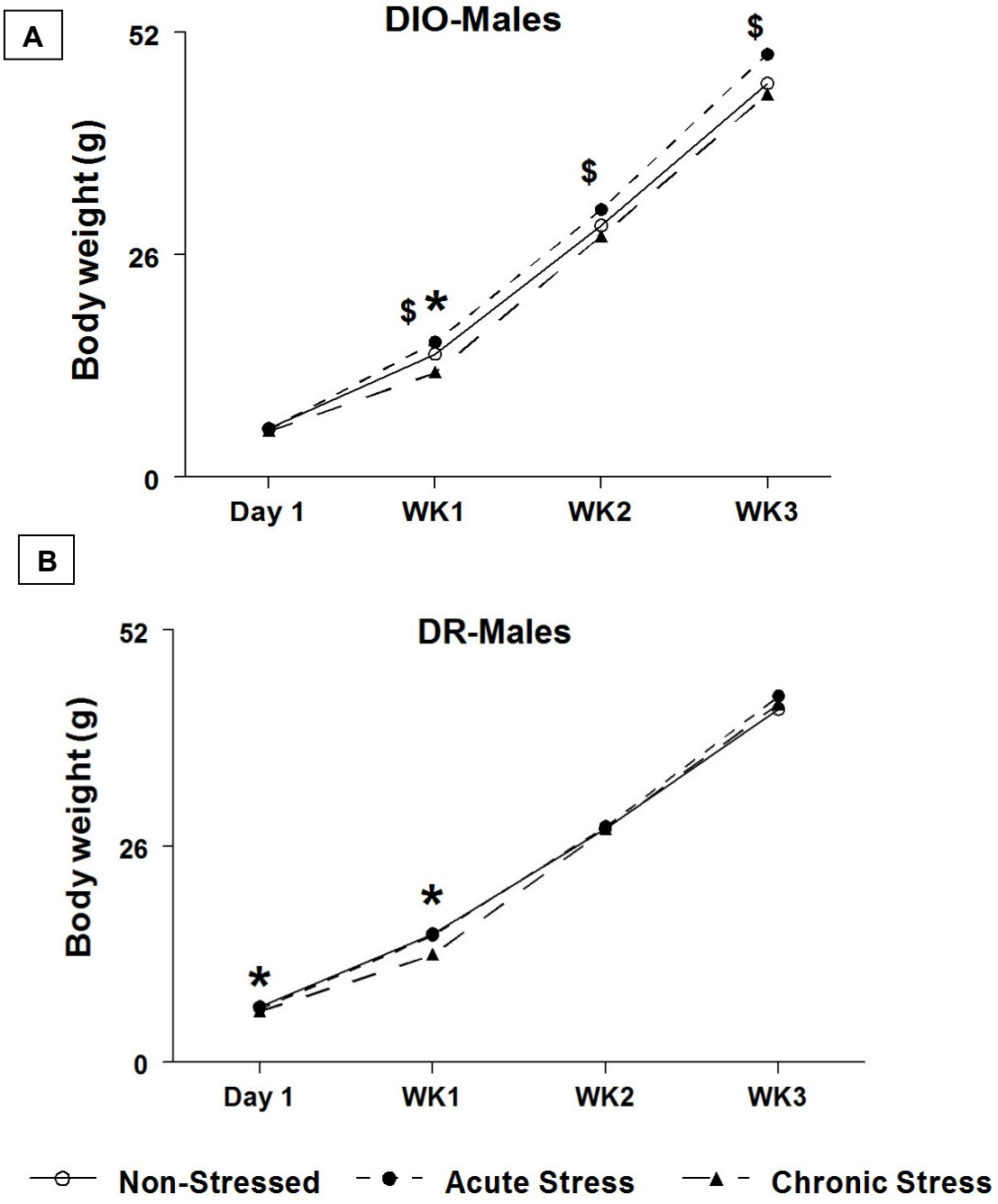

Fig. 4-2 A and B: Effects of prenatal stress on the body weight of male DIO and DR offspring until weaning

Body weight (means±SE, g) of male DIO and DR offspring whose dams underwent normal gestation (non-stressed), acute stress (surgical stress once a week during gestation) or chronic restraint stress (thrice daily from day 14-21 of gestation) from birth until weaning at 3 weeks of age are shown in Fig 4-2 A and B respectively. '*' $p<0.05$ chronic stress vs non-stressed group. '$' $p<0.05$ acute stress vs non-stressed group.

Post-weaning BW gain

Post weaning BW gain (means±SE, g) for all the groups were represented as averages of the values obtained from subtracting the weaning weight (week 3) from the weekly body weight. BW gain from weaning to week 9 of the male DIO and DR groups are shown in Fig. 4-3. In DIO animals, the effects of chronic prenatal stress persisted for 2 weeks after weaning, where DIO-CS animals had significantly lower BW gain in week 4 (34.7±0.6) and week 5 (82.8±1.4) compared to the controls (DIO-NS; 36.9±0.7 and 89.5±1.5 on weeks 4 and 5 respectively; $p<0.05$). After week 5, DIO-CS animals had caught up and their BW gain was comparable to the controls. As observed in the pre-weaning period, prenatal acute stress significantly increased the BW gain in the DIO animals from week 4 (40.1±0.4) through week 9 (319.7±2.9) compared to controls (36.9±0.7 and 303.7±5.6 at weeks 4 and 9 respectively; $p<0.05$). Interestingly, the effects of prenatal stress (both acute and chronic) were not observed in DR male offspring, suggesting that DR's are resistant to the effects of prenatal stress compared to DIO's. Taken together, these results indicate that there is an overall genotype, prenatal stress and genotype-prenatal stress interaction with respect to postnatal weight gain on chow diet in the male offspring.

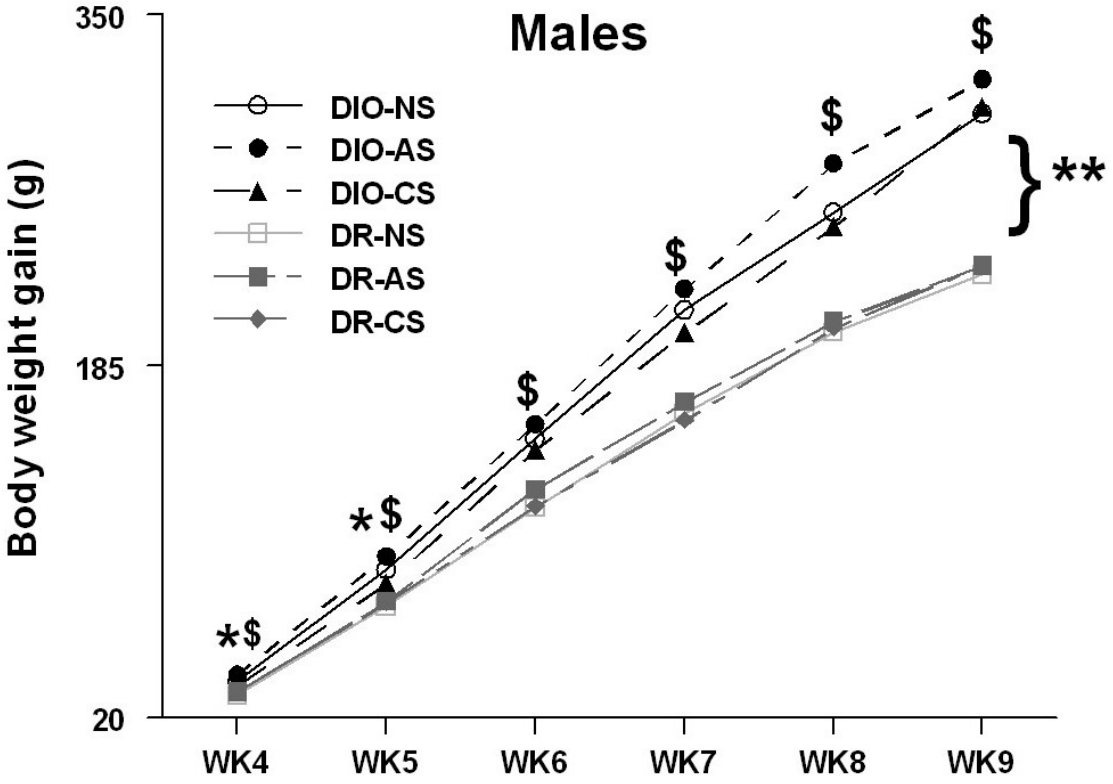

Fig. 4-3: Effects of prenatal stress on the post-weaning body weight gain in the male DIO and DR offspring

Post weaning body weight gain (means±SE, g) of male DIO and DR offspring from week 4 through week 9 is shown above. NS stands for offspring from non-stressed dams, AS for offspring from dams who underwent acute stress during pregnancy and CS for offspring from dams who underwent chronic restraint stress during pregnancy. '*' $p<0.05$ DIO-CS vs DIO-NS, '$' $p<0.05$ DIO-AS vs DIO-NS and '**' denotes significant differences ($p<0.05$) between DIO and DR groups at all time points.

Calorie intake

Calorie intake (means±SE, kcal) for each week was calculated by multiplying the average food intake per week by the calorie content of the chow diet (3.11kcal/g). Calorie intake in the post weaning period from week 4 to week 9 in the male offspring is represented in Fig. 4-4. Supporting the BW gain data, the average calorie intake was significantly lower in DIO-CS animals in week 4 (215.7±5.3 vs 231.3±3.1 in control, $p<0.05$) and week 5 (348.3±9.1 vs 380.6±7.5 in control, $p<0.05$). After week 5, no differences in calorie intake were observed between DIO-CS and DIO-NS animals. On the other hand, DIO-AS animals had higher calorie intake relative to controls on weeks 7 (785.6±47.6 vs 602.8±44.4 in control, $p<0.05$) and week 8 (768.4±26.9 vs 691.6±17.6 in control, $p<0.05$). A similar trend was observed in DR animals. There was a significant effect of genotype, prenatal stress and genotype-prenatal stress interaction on calorie intake in DIO and DR animals.

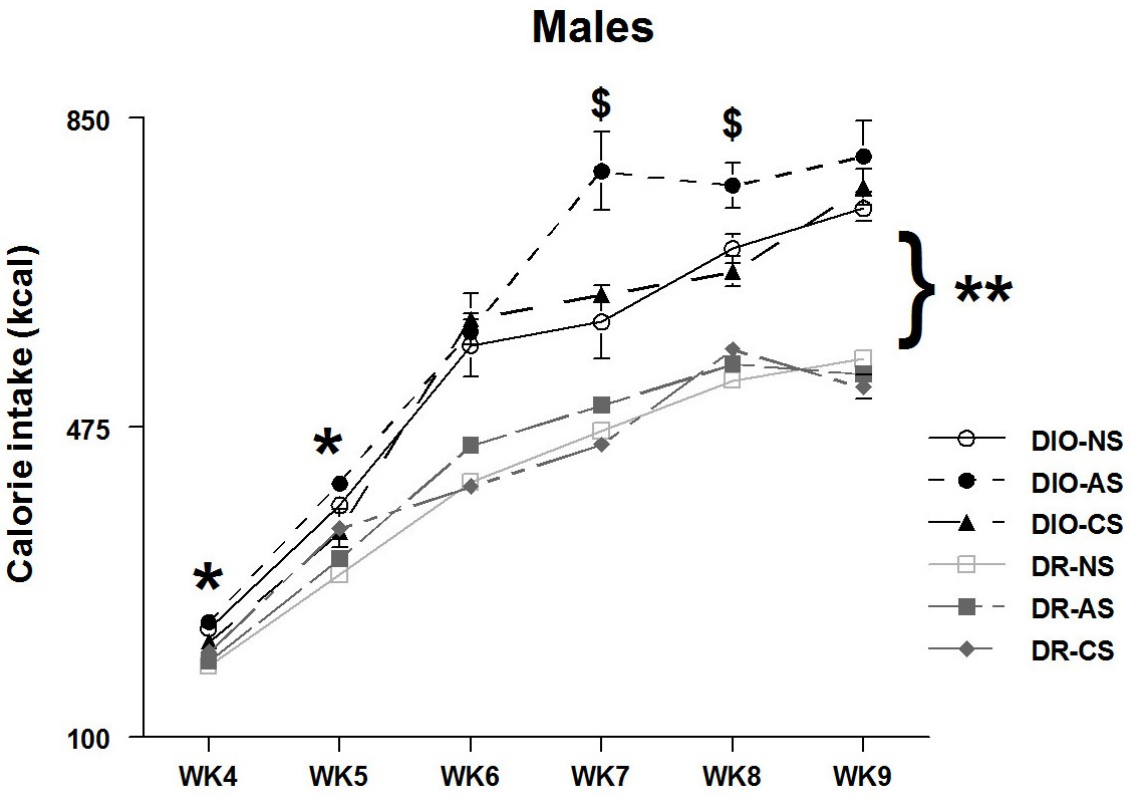

Fig 4-4: Effects of prenatal stress on the post-weaning calorie intake in the male DIO and DR offspring

Post weaning calorie intake (means±SE, kcal) of the male DIO and DR offspring from week 4 through week 9 is shown above. NS-Offspring from non-stressed dams, AS-Offspring from dams who underwent acute stress during pregnancy and CS-Offspring from dams who underwent chronic restraint stress during pregnancy. '*' denotes $p<0.05$ between CS and NS groups in both genotypes, '$' $p<0.05$ between AS and NS in both genotypes and '**' denotes significant differences ($p<0.05$) between DIO and DR groups at all time points.

BW gain, Fat mass, Leptin and Triglyceride levels after 1 week HF diet exposure

Final BW (means±SE; g), BW gain/week (means±SE; g), average calorie intake per week (kcal), Visceral adipose tissue (VAT) mass (means±SE; g), VAT to BW ratio (%), serum leptin (means±SE; ng/ml) and serum triglyceride (means±SE; mg/dl) levels after one week HF diet exposure in DIO and DR male offspring are shown in Table 1 and 2 respectively. In the DIO-NS offspring, exposure to 1 wk of HF diet did not change the final BW compared to chow-fed DIO-NS offspring (408.9±8.4 vs 387.2±11.8). However, in DIO-AS (433.3±7.7 (HF) vs 407.1±4.9 in Chow fed, $p<0.05$) and DIO-CS (415.9±7.4 (HF) vs 372.2±4.2 (Chow fed), $p<0.05$) offspring, exposure to 1 wk HF diet resulted in a significant increase in the final BW compared to their chow-fed counterparts. The final BW in DIO-AS HF-fed group was significantly greater than the DIO-NS HF fed group. Two-way ANOVA analysis revealed a significant stress ($p=0.004$) and diet ($p<0.0001$) effect on the final BW in the DIO offspring. In the DR offspring, no changes were observed in the final BW between the groups after exposure to 1 wk HF diet.

BW gain per week

BW gain (means±SE; g) after 1 week HF diet exposure was calculated by subtracting the body weight measured at the beginning of HF diet exposure (Week 9) from the final body weight. In the DIO offspring, exposure to 1 wk HF diet exposure significantly increased BW gain/week in DIO-NS (56.1±2.0 vs 40.9±4.3 in chow fed, $p<0.05$), DIO-AS (66.3±2.6 vs 39.4±2.3 in chow fed, $p<0.05$) and DIO-CS (57.1±3.4 vs 29.5±2.8 in chow fed, $p<0.05$). The BW gain in the DIO-AS HF-fed group was significantly higher than the DIO-NS HF-fed group (66.3±2.3 vs 56.1±2.0 respectively,

$p<0.05$). In the DR offspring, no changes were observed in the BW gain/week between the groups after exposure to 1 wk of HF diet. In both DIO and DR offspring, there was a significant stress ($p<0.05$) and diet ($p<0.05$) effect in increasing BW gain per week.

Average Calorie intake per week

Average calorie intake per week (means±SE; kcal) was calculated by multiplying the total food intake of the animal for the week by the amount of calories per g of food (3.11 kcal/g of chow diet and 4.73 kcal/g of HF diet). In the DIO offspring, exposure to 1 wk of HF diet significantly increased the calorie intake in DIO-NS (946.2±24.0 vs 732.3±57.2 (Chow), $p<0.05$), DIO-AS (1216.0±121.0 vs 677.2±35.6 (chow), $p<0.05$) and DIO-CS (998.5±34.0 vs 656.7±30.6 (chow), $p<0.05$) relative to their respective chow-fed groups. The average calorie intake in the HF-fed DIO-AS group was significantly ($p<0.05$) greater than the HF-fed DIO-NS group. A similar pattern was observed in the DR groups where exposure to HF diet significantly increased the calorie intake in all the stressed and non-stressed groups when compared to their chow-fed counterparts. In both DR and DIO offspring, a significant effect of diet and diet-stress interaction was observed with respect to calorie intake.

Total visceral adipose tissue (VAT) mass and VAT/BW ratio

Upon sacrifice at the end of 1 week of HF diet exposure, VAT was collected, weighed and the data are represented as total VAT mass (means±SE, g) and VAT/BW ratio (correcting for the differences in the body weight (means±SE, %) in Table 1 and 2. The total VAT mass and the VAT/BW ratio were significantly higher in the HF fed DIO and DR groups when compared with their chow-fed counterparts. Interestingly, the chow-fed DIO-AS offspring had higher VAT mass when compared with the DIO-NS

chow groups. In the DR animals, the VAT/BW ratio in the DR-CS group (1.3±0.1, $p<0.05$) after 1 wk HF diet exposure was significantly lower compared to their non-stressed counterparts (1.6±0.1). A significant stress ($p<0.05$) and diet ($p<0.05$) effect was observed in both DIO and DR offspring with respect to VAT/BW ratio. However, there was no significant interaction between stress and diet.

Serum Leptin

Serum leptin levels (means±SE, ng/ml) measured at the end of 1 week HF diet exposure in DIO and DR offspring are tabulated in tables 1 and 2 respectively. Serum leptin levels were significantly higher in the HF-fed DIO-NS (53.3±7.1 VS 11.6±1.8 (chow), $p<0.05$), DIO-AS (60.2±8.4 vs 10.5±1.4 (chow), $p<0.05$) and DIO-CS (53.0±5.6 vs 5.8±0.6 (chow), $p<0.05$) compared with the chow-fed groups. Two-way ANOVA analysis revealed a significant diet but not stress effect with respect to leptin levelsin DIO animals. However, in DR animals, in addition to the diet effect, a significant stress effect was also observed. HF diet-induced increases in serum leptin levels were significantly ($p<0.05$) greater in the DR-AS group when compared to that in the DR-NS offspring.

Serum Triglyceride

HF diet exposure for 1 week resulted in a significant increases in the serum triglyceride levels (means±SE, mg/dl) in both the non-stressed and prenatally stressed (both acute and chronic stressed) DIO and DR groups compared to their chow-fed counterparts. This is presented in tables 1 and 2. We observed a significant diet but not stress effect with respect to serum triglyceride levels in both the DIO and DR offspring.

Parameters	DIO-NS		DIO-AS		DIO-CS		Significance		
	CHOW	HF	CHOW	HF	CHOW	HF	Stress	Diet	SXD
Final BW (g)	387.2±11.8	408.9±8.4	407.1±4.9	433.3±7.7 *$	372.2±4.2	415.9±7.4 *	0.004	<0.001	0.41
BW gain/wk (g)	40.9±4.3	56.1±2.0 *	39.4±2.3	66.3±2.6 *$	29.5±2.8	57.1±3.4 *	0.01	<0.001	0.07
Avg. Calorie intake/wk (kcal)	732.3±57.2	946.2±24 *	677.2±36	1216±121 *$	656.7±30.6	998.5±34 *	0.15	<0.001	0.05
Total VAT mass (g)	4.2±0.5	7.7±0.5 *	5.7±0.5#	8.6±0.5 *	4±0.3	8±0.3 *	0.01	<0.001	0.5
BW ratio (%)	1.1±0.1	1.9±0.1 *	1.4±0.1	2.0±0.1 *	1.1±0.1	2.0±0.1 *	0.05	<0.001	0.3
Leptin (ng/ml)	11.6±1.8	53.3±7.1 *	10.5±1.4	60.2±8.4 *	5.8±0.6	53.0±5.6 *	0.50	<0.001	0.8
Triglycerides (mg/dl)	205.6±17	498.8±23 *	203.5±14.2	497.9±20 *	162.8±7.9	481.5±18 *	0.16	<0.001	0.5

Table 1: Effects of prenatal stress on the final BW (g), BW gain (g), average calorie intake per week (kcal), Visceral adipose tissue (VAT) mass (g), VAT to BW ratio (%), Leptin (ng/ml) and Triglyceride (mg/dl) levels after one week HF diet exposure in the DIO male offspring are shown above in the table. NS-Offspring from non-stressed dams, AS- Offspring from dams who underwent acute stress during pregnancy and CS-Offspring from dams who underwent chronic restraint stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow groups, $ denotes significant difference ($p<0.05$) from DIO-NS chow group and # denotes significant difference ($p<0.05$) from all chow groups.

Parameters	DR-NS		DR-AS		DR-CS		Significance		
	CHOW	HF	CHOW	HF	CHOW	HF	Stress	Diet	SXD
Final BW (g)	293.2±7.1	305.3±3.9	302.6±5.0	307.9±3.0	293.3±4.7	302.9±5.9	0.29	0.03	0.79
BW gain/wk (g)	28.02±1.6	31.9±2.6	28.8±1.2	30.2±2.2	19.7±1.7#	25.3±1.6	0.0001	0.01	0.48
Avg. Calorie intake/wk	475.4±14.2	651.1±4.0*	595±25.4#	674.4±21.9*	503.4±20.8	662±11.1*	0.001	<0.001	0.03
Total VAT mass (g)	2.8±0.2	4.8±0.3*	3.0±0.2	4.7±0.2*	2.6±0.2	4.4±0.3*	0.3	<0.001	0.8
VAT/BW ratio (%)	0.96±0.1	1.6±0.1*	0.99±0.1	1.5±0.1*	0.89±0.1	1.3±0.1*$	0.04	<0.001	0.4
Leptin (ng/ml)	4.3±0.4	10.3±1.8*	4.7±0.2	13.3±1.2*$	2.9±0.3	7.8±0.8*	0.007	<0.001	0.09
Triglycerides (mg/dl)	76.7±6.1	295.6±46*	98.1±13.4	293.7±28*	81.9±9.7	312.9±49*	0.5	<0.001	0.8

Table 2: Effects of prenatal stress on the final BW (g), BW gain (g), average calorie intake per week (kcal), Visceral adipose tissue (VAT) mass (g), VAT to BW ratio (%), Leptin (ng/ml) and Triglyceride (mg/dl) levels after one week of HF diet exposure in the DR male offspring are shown above. NS indicates offspring from non-stressed dams, AS- Offspring from dams who underwent acute stress during pregnancy and CS-Offspring from dams who underwent chronic restraint stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow groups, $ denotes significant difference ($p<0.05$) from all HF groups and # denotes significant difference ($p<0.05$) from all chow groups.

Blood glucose levels:

In order to assess glucose homeostasis in prenatally stressed DIO and DR offspring, blood glucose, serum insulin and C-peptide levels were measured after 1 week chow or HF diet exposure. Blood glucose levels (means±SE, mg/dl) were measured from the trunk blood at the time of sacrifice in the DIO and DR male offspring. Data from the acute and chronic stress groups are represented separately in Fig. 4-5 and Fig. 4-6 respectively. HF diet exposure for 1 week significantly increased blood glucose levels in all the groups: DIO-NS (116.1±3.3 vs 103.1±4.2 (chow), $p<0.05$), DIO-AS (118.0±4.4 vs 99.3±4.1 (chow), $p<0.05$) and DIO-CS (127.7±6.9 vs 106.0±2.2 (chow), $p<0.05$) compared to their chow-fed counterparts (Fig. 4-5A and 4-6A). We observed a similar dietary effect in DR animals where HF-fed animals in all the groups had an increase in their blood glucose levels compared to their chow-fed controls (Fig. 4-5B and 4-6B). Two-way ANOVA analysis revealed a significant ($p<0.05$) diet but not prenatal stress effect on blood glucose levels in both DIO and DR animals.

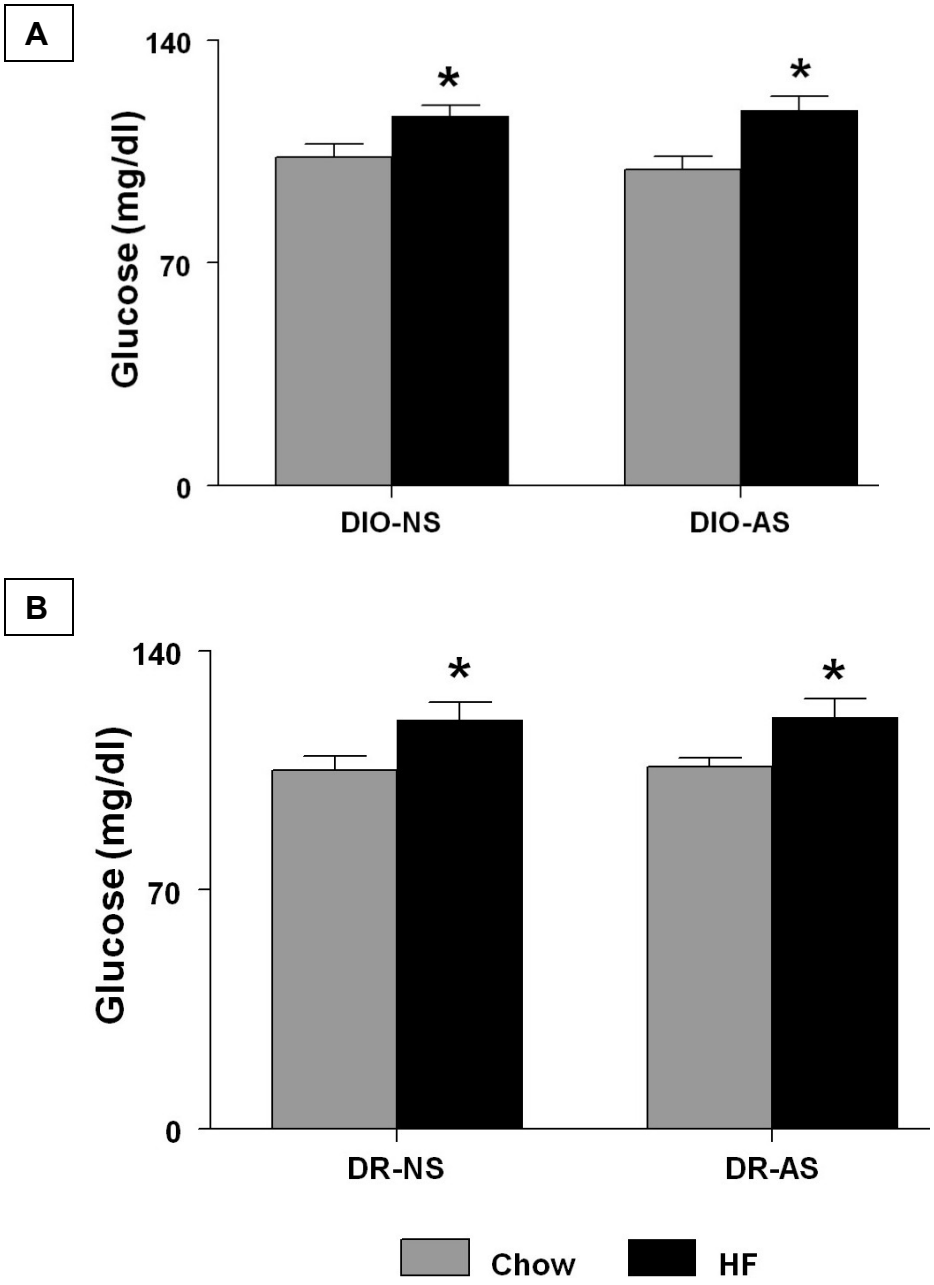

Fig 4-5: Effects of prenatal acute stress and postnatal HF diet exposure for 1 week on blood glucose levels in the DIO and DR male offspring

Blood glucose levels (means±SE, mg/dl) of the prenatally stressed (acute stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 4-5A and B respectively. NS -Offspring from non-stressed dams, AS-Offspring from dams who underwent acute stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow-fed groups.

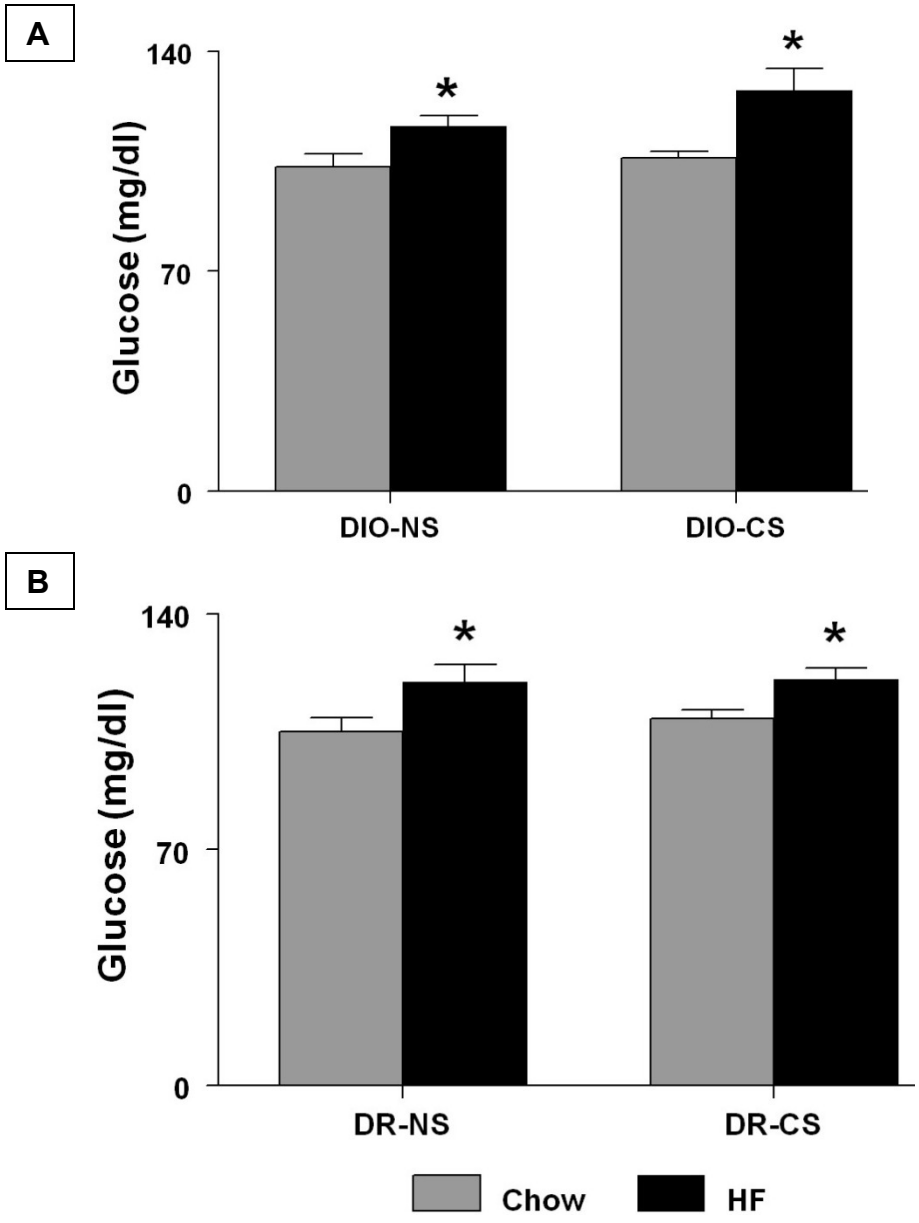

Fig. 4-6 A and B: Effects of prenatal chronic stress and postnatal HF diet exposure for 1 week on blood glucose levels in the DIO and DR male offspring

Blood glucose levels (means±SE, mg/dl) of the prenatally stressed (chronic stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 4-6A and B respectively. NS-Offspring from non-stressed dams, CS-Offspring from dams who underwent chronic restraint stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow-fed groups.

Serum insulin levels:

Serum insulin levels (means±SE, ng/ml) in the acute and chronic stress DIO and DR male offspring after exposure to 1 week of chow or HF diet are shown separately in Figs. 4-7 and 4-8 respectively. HF diet exposure for 1 week significantly increased serum insulin levels in the DIO-AS (2.041±0.2 (HF) vs 8.5±1.6 (chow), $p<0.05$) and DIO-CS (1.8±0.4 (HF) vs 9.0±2.3 (chow), $p<0.05$) compared to their chow-fed counterparts (Fig. 4-7A and 4-8A). While we observed a trend towards an increase in serum insulin levels in the HF-fed DIO-NS group (3.8±0.7) compared to chow-fed controls (1.35±0.2), it did not attain statistical significance. Also, the increase in the serum insulin levels observed in the acute and chronic prenatal stress DIO offspring after 1 week HF diet exposure was significantly greater than observed in the HF-fed non-stressed offspring (both chow and HF-fed groups). Two-way ANOVA analysis revealed a significant stress, diet and stress-diet interaction with respect to serum insulin levels in the DIO male offspring.

In the DR offspring, HF diet exposure for 1 week resulted in an increase in the serum insulin levels only in the non-stressed group (3.0±0.5 (HF) vs 1.5±0.3 (chow), $p<0.05$), but not in the acute or chronic stress groups. There was a significant effect of diet but not stress in the DR offspring with respect to serum insulin levels.

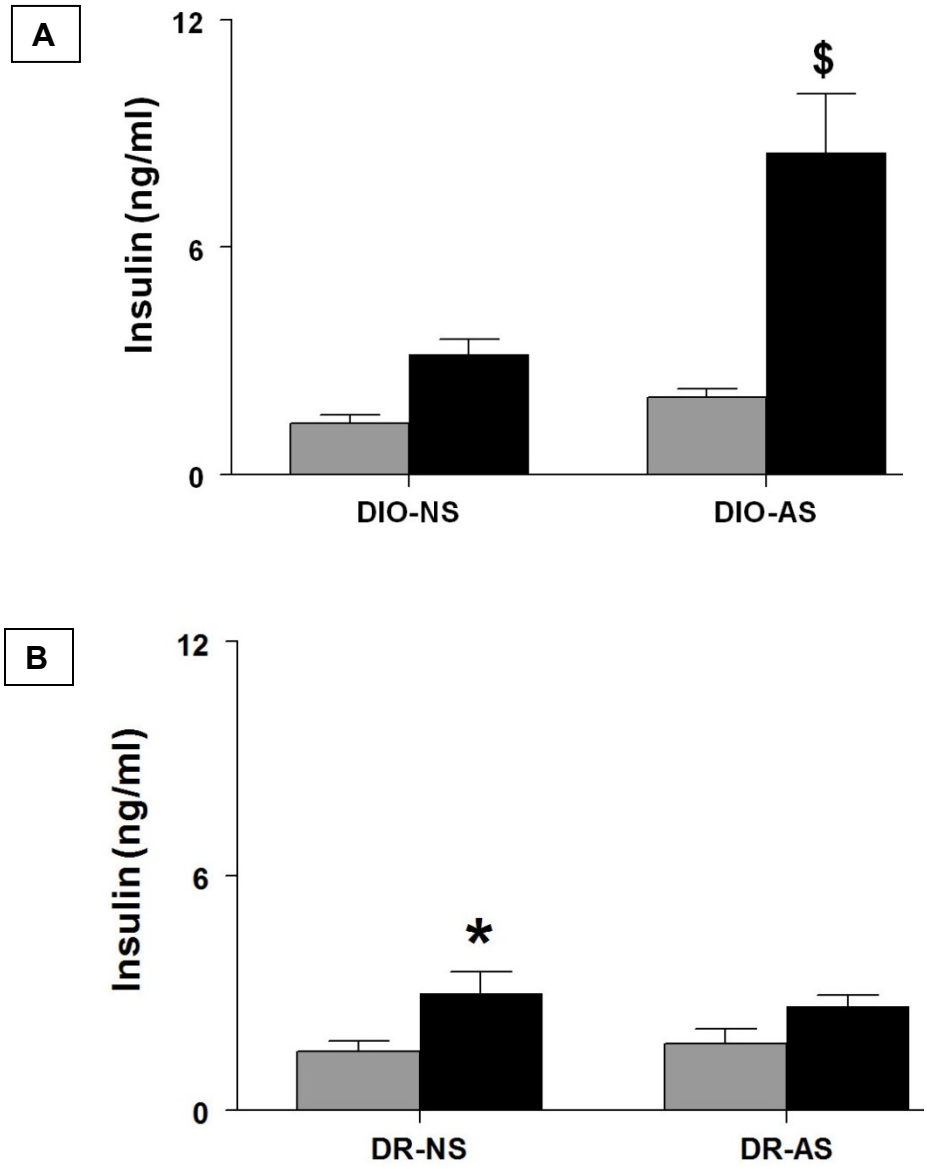

Fig 4-7: Effects of prenatal acute stress and postnatal HF diet exposure for 1 week on serum insulin levels in the DIO and DR male offspring

Serum insulin levels (means±SE, ng/ml) of the prenatally stressed (acute stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 4-7A and B respectively. NS -Offspring from non-stressed dams, AS-Offspring from dams who underwent acute stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow-fed group. $ denotes significant difference ($p<0.05$) from all the other groups.

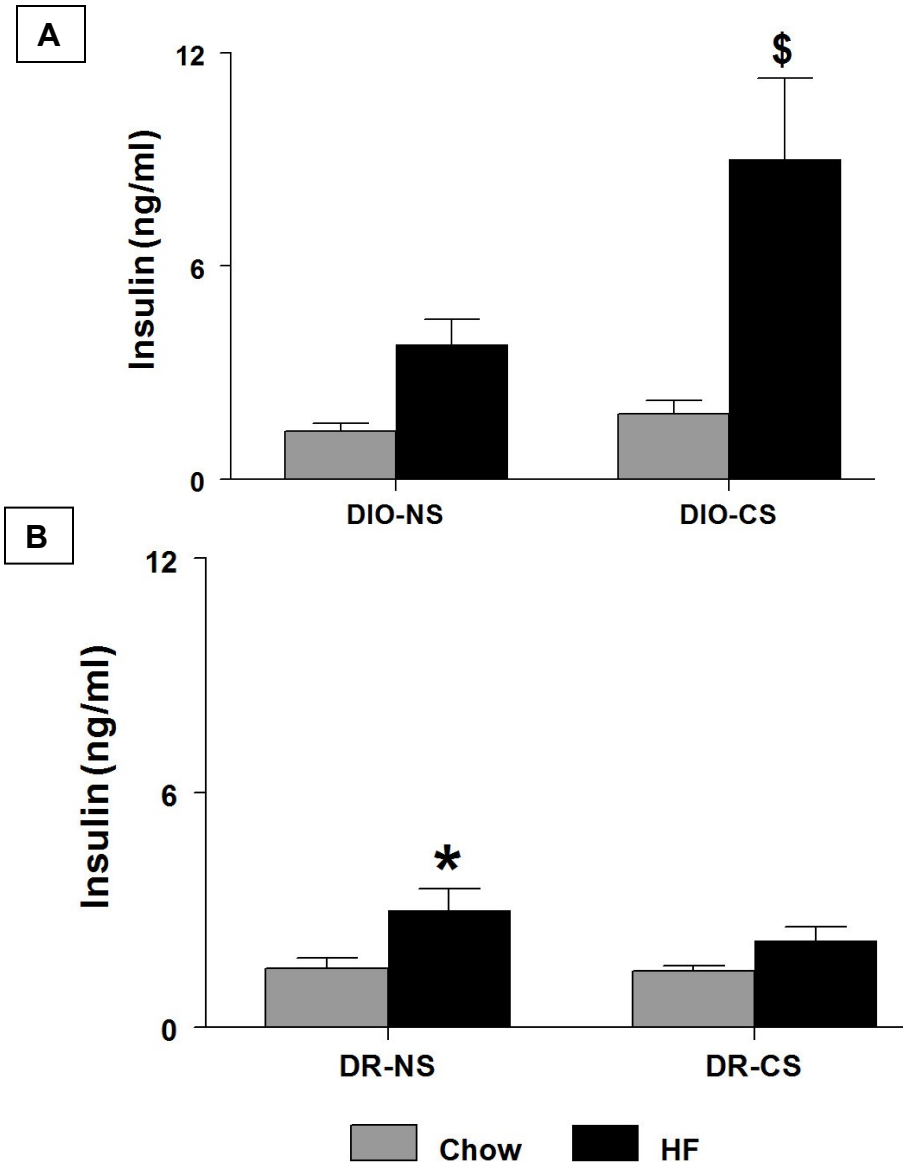

Fig 4-8: Effects of prenatal chronic stress and postnatal HF diet exposure for 1 week on serum insulin levels in the DIO and DR male offspring

Serum insulin levels (means±SE, ng/ml) of the prenatally stressed (chronic stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 4-8A and B respectively. NS-Offspring from non-stressed dams, CS-Offspring from dams who underwent chronic restraint stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow-fed group. $ denotes significant difference ($p<0.05$) from all the other groups.

Serum C-peptide levels:

C-peptide levels (means±SE, nM/ml) were measured in the serum separated from the trunk blood at the time of sacrifice after exposure to 1 week chow or HF diet in the DIO and DR male offspring. Data from the acute and chronic stress groups are represented separately in Fig. 4-9 and Fig. 4-10 respectively. HF diet for 1 week caused a significant increase in the serum C-peptide levels in the DIO-NS (3.25±1.9 (HF) vs 1.7±2.6 (Chow), $p<0.05$), DIO-AS (4.4±5.6 (HF) vs 1.9±2.5 (Chow), $p<0.05$) and DIO-CS groups (4.3±6.1 (HF) vs 1.9±0.3 (Chow), $p<0.05$) (Fig. 9-A and 10-A). There were no differences in C-peptide levels among the chow-fed animals between the stressed groups.

In the DR male offspring, HF diet-induced increase in C-peptide levels were observed only in the DR-NS and DR-AS groups (Fig. 9-B). In the chronic stress group, 1 week HF diet exposure did not cause any change in the C-peptide levels (Fig. 10-B). Diet had an overall effect ($p<0.05$) in the increase in the C-peptide levels observed among the DIO and DR groups.

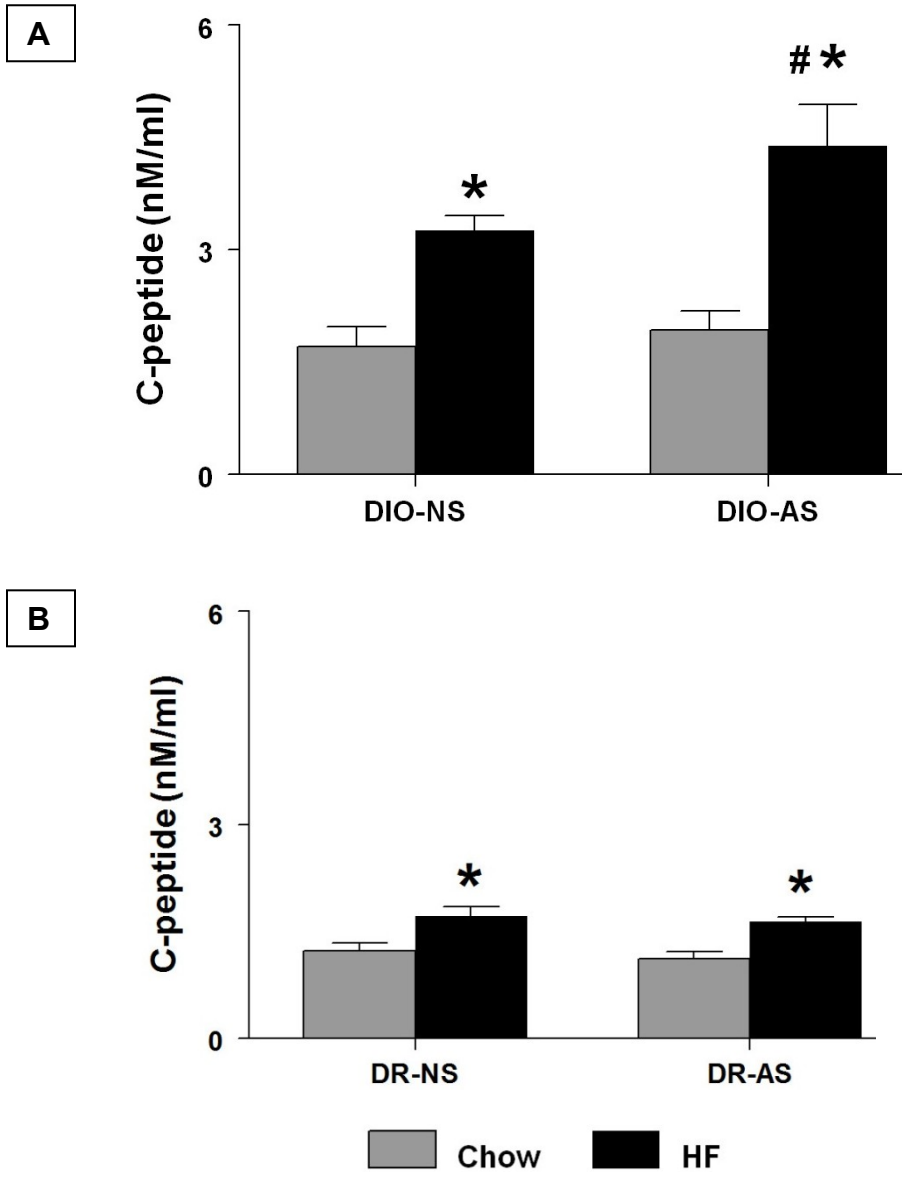

Fig 4-9: Effects of acute prenatal stress and postnatal HF diet exposure for 1 week on serum C-peptide levels in the DIO and DR male offspring

Serum C-peptide levels (means±SE, nM/ml) of the prenatally stressed (acute stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 4-9A and B respectively. NS-Offspring from non-stressed dams, AS-Offspring from dams subjected to acute stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow-fed group. '#' denotes significant difference ($p<0.05$) from chow-fed DIO-NS group.

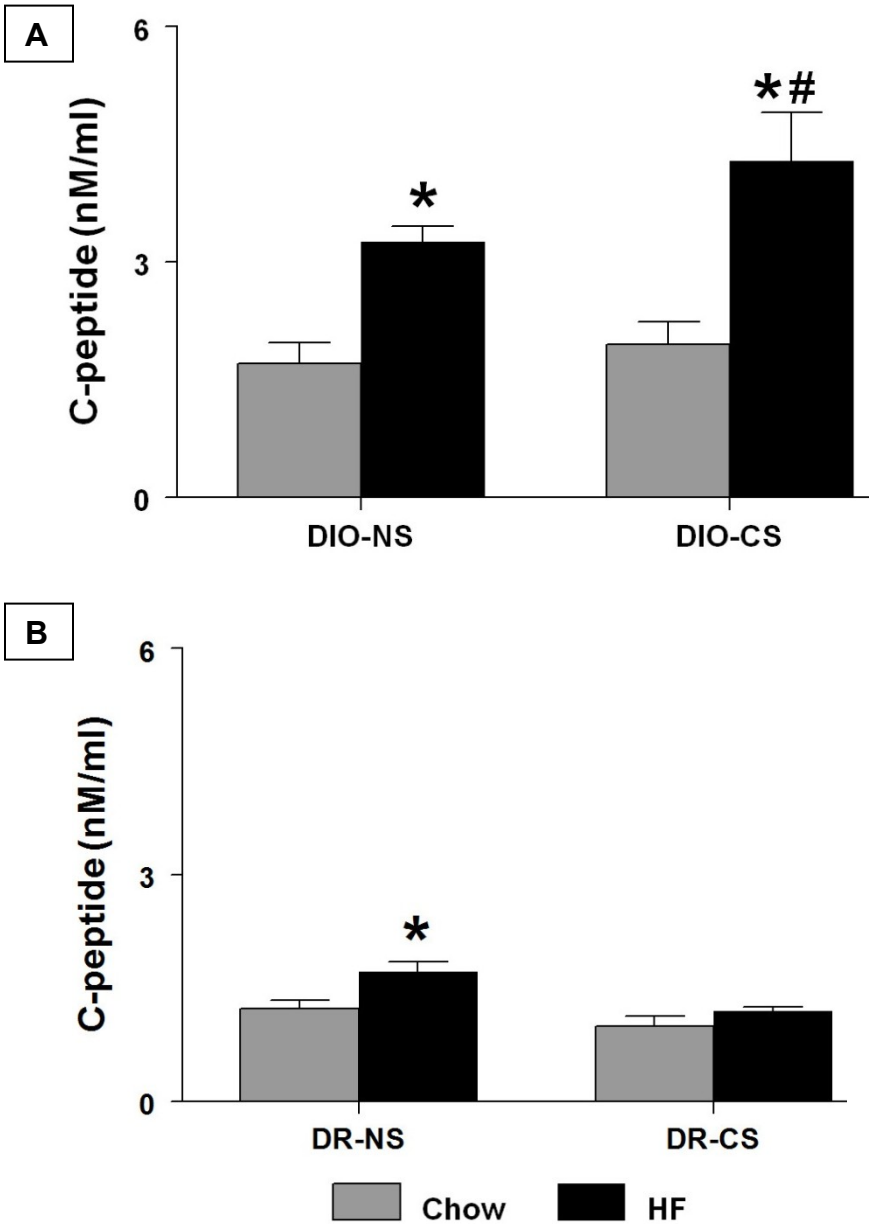

Fig 4-10: Effects of chronic prenatal stress and postnatal HF diet exposure for 1 week on serum C-peptide levels in DIO and DR male offspring

Serum C-peptide levels (means±SE, nM/ml) of the prenatally stressed (chronic stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 4-10A and B respectively. NS-Offspring from non-stressed dams, CS-Offspring from dams who underwent chronic restraint stress during pregnancy. * denotes significant difference ($p<0.05$) from their respective chow-fed group. '#' denotes significant difference ($p<0.05$) from chow-fed DIO-NS group.

3. Discussion:

It is now increasingly recognized that intrauterine perturbations may have long-term health consequences in the offspring. Several epidemiological studies have associated compromised *in utero* environment, as evidenced by low birth weight in the offspring, with increased incidence of obesity, type 2 diabetes and hypertension during adulthood. With escalating rates of maternal obesity, it is important to understand if pre-existing genetic predisposition to obesity would alter the offspring's susceptibility to adverse *in utero* programming factors. Hence, we investigated the effects of prenatal stress (acute and chronic) on several metabolic parameters in the offspring in the background of maternal obesity using DIO and DR animal model.

Results from the present study demonstrate that prenatal stress can predispose the offspring to adverse metabolic outcomes especially after postnatal dietary challenge if there is genetic predisposition to obesity. Also, acute and chronic prenatal stress resulted in different phenotypes in the offspring. With acute stress, DIO offspring exhibited increase in postnatal BW gain, calorie intake and fat mass. Upon HF diet challenge, these animals were hyperinsulinemic and had high circulating C-peptide levels. On the other hand, chronic stress resulted in reduced birth weight, reduced postnatal BW gain and calorie intake until 5 weeks of age. After 5 weeks of age, DIO offspring subjected to chronic prenatal stress showed catch-up growth and their BW gain and calorie intake were comparable to the non-stressed controls. HF diet challenge resulted in hyperinsulinemia and higher C-peptide levels in these animals as well. These changes were independent of any changes in BW or fat mass in the DIO

offspring. DR offspring were resistant to the adverse metabolic consequences of prenatal stress with postnatal HF diet challenge.

Consistent with previous findings, our results demonstrated that prenatal stress caused intrauterine growth retardation as indicated by a reduction in the birth weight in male offspring. However, the effect of prenatal stress on birth weight varied depending on the type of stressor and genotype. In the present study, chronic but not acute prenatal stress decreased birth weight in the DR male offspring. The reason for this differential effect on birth weight of chronic versus acute stress may be due to the fact that the animals in the chronic stress group were stressed during the last week of gestation, which is a period of rapid growth in fetal life. Also, previous studies have suggested that restraint stress during the last week of gestation reduces food intake in rats [179], thus the chronic stress procedure mimics maternal under-nutrition resulting in low birth weight. Although, it is difficult to separate the effects of under-nutrition and stress (or glucocorticoids) per se in this model, adrenal gland hormones do play a role in this phenomenon as adrenalectomy in the dam prevents prenatal stress-induced reduction in birth weight [180].

Interestingly, DIO animals, irrespective of stress, had lower birth weight compared to DR offspring. Prenatal stress, both acute and chronic did not lower this further, most probably because it would not have been compatible with survival. To our knowledge, this is the first time birth weight has been reported in DIO and DR rats. Low birth weight observed in animals genetically predisposed to obesity further supports the idea of low birth weight being a predisposing factor for obesity in the DIO model. Though many studies have reported a similar observation on prenatal stress-induced

reduction in birth weight [181-184], few studies have also reported no change [185, 186] or increase in birth weight [169] after prenatal stress exposure. The difference in the experimental protocol in terms of the type, intensity and the timing of stress procedure during gestation could explain the disparity in the findings between studies.

DIO males subjected to prenatal acute stress gained significantly more body weight in the pre-weaning period (starting from postnatal week 1) and in the post-weaning period. Increase in calorie intake observed in these animals could be partly responsible for this increase in postnatal BW gain. Studies suggest that prenatal stress could program the feeding circuits of the offspring *in utero* for hyperphagia during the immediate postnatal period. Warnes et al have reported up-regulation of neuropeptide-Y (NPY) gene expression in the fetal arcuate nucleus in response to maternal glucocorticoids or under-nutrition during pregnancy [187]. Furthermore, acutely stressed DIO offspring were more sensitive to HF diet challenge in terms of higher BW gain and calorie intake compared to the non-stressed offspring. Increase in white adipose tissue suggests that BW gain was primarily due to adiposity. On the other hand, DR offspring exposed to acute prenatal stress were able to maintain normal body weight despite an increase in calorie intake. These suggest that acute prenatal stress makes the DIO offspring more susceptible to postnatal metabolic challenges than DR offspring.

In contrast to acute stress, DIO and DR offspring exposed to chronic prenatal stress were able to maintain normal adult body weight and calorie intake. Also, chronic prenatal stress did not affect the offspring's response to 1 week HF diet challenge in terms of body weight, food intake, fat mass, and leptin and triglyceride levels. This

finding is in accordance with previous studies where prenatal stress did not affect adult body weight and other metabolic parameters [181, 188] [176]. In fact, chronic prenatal stress attenuated the increase in visceral adipose tissue mass in the DR offspring. It should be noted that all the measurements were made at a young age (10 weeks) and also the postnatal HF diet exposure was only for a short duration. There is every possibility that obesity may emerge at a later point in adulthood or after chronic exposure to HF diet.

While examining glucose homeostasis in the offspring exposed to prenatal stress, we observed no difference in basal glucose, insulin or C-peptide levels in the DIO and DR offspring on chow. However, when exposed to 1 week HF diet, we were able to observe a more pronounced effect of both acute and chronic prenatal stress on serum insulin levels in DIO offspring. In DR animals, chronic prenatal stress prevented the HF diet-induced increase in serum insulin levels and C-peptide levels. Although, HF diet treatment for 1 week *per se* increased blood glucose levels in all the groups, prenatal stress did not have any additive effect. A previous report demonstrating changes in basal glucose levels due to prenatal stress were carried out in animals older (5 months old) than the ones used in our present study [189]. In another study, prenatal stress-induced increases in blood glucose levels were observed only after 3-4 months of HF diet treatment [190]. However, these animals did not have any genetic propensity to develop obesity. Hence, age, genetic background and duration of postnatal HF diet exposure are important determinants in assessing glucose homeostasis in the prenatally stressed offspring. It is likely that these animals are insulin resistant. Euglycemic clamp experiments are needed to further determine insulin

sensitivity in the prenatally stressed offspring. However, the presence of higher C-peptide and insulin levels suggest that they are likely to be insulin resistant. The hyperinsulinemia could also be due to reduced clearance of insulin from the system. Carcinoembryonic antigen related cell adhesion molecule-1 (CEACAM-1) is a protein in the liver that promotes insulin clearance [191]. A reduction in CEACAM-1 levels could potentially lead to hyperinsulinemia [192].

Overall, the present study suggests that prenatal stress could program offspring for adverse metabolic outcomes especially when they are challenged with HF diet in the postnatal period. Pre-existing genetic predisposition to obesity increased the susceptibility to the metabolic programming effects of prenatal stress. In order to completely understand the phenotype of the prenatally stressed offspring, we need to extend these studies by increasing the duration of HF diet exposure during postnatal life.

CHAPTER 5

EFFECTS OF PRENATAL STRESS ON STRESS AXIS FUNCTION IN DIO AND DR RATS

Introduction

Growing evidence from animal and human epidemiological studies supports the idea that prenatal stress is not only associated with increased susceptibility to develop psychological disorders but also metabolic disorders like obesity and diabetes [169, 172]. Results described in the previous chapter also suggest that prenatal stress combined with postnatal HF feeding could predispose the offspring to adverse metabolic outcomes like obesity and hyperinsulinemia. In addition, our results also suggest that individuals with pre-existing genetic predisposition to obesity are more susceptible to the programming effects of prenatal stress. Though the association between prenatal stress and metabolic disorders is well established, the mechanism behind this phenomenon is far from clear. Dysregulation of stress axis function could be one of the possible mechanisms through which prenatal stress programs the offspring for adult diseases.

The association between abnormal stress axis activity or cortisol levels with obesity emerged from the clinical similarities between Cushing's diseases and metabolic syndrome (abdominal obesity, insulin resistance and hypertension) [193, 194]. Stress axis or the hypothalamo pituitary adrenal (HPA) axis comprises of 3 parts: the hypothalamus, the pituitary and the adrenal gland. Stress axis activation commences with the activation of corticotropin releasing hormone (CRH) neurons in the paraventricular nucleus (PVN) of the hypothalamus. Though a variety of neurotransmitters like 5-HT and GABA play an important role in modulating CRH neuronal activation, norepinephrine (NE) from the brain stem nuclei provides the primary stimulus for CRH synthesis and release [195]. Upon activation, CRH is released

in the median eminence and then transported to the pituitary through the hypothalamo-hypophyseal portal system. In the pituitary, CRH acts on the corticotrophs to release adrenocorticotropin (ACTH). ACTH enters the systemic circulation and acts on the adrenal cortex to release glucocorticoids (cortisol in humans and corticosterone (CORT) in rodents). Once released into the circulation, glucocorticoids bind to their receptors (GR) in higher centers like hippocampus and exert a negative feedback effect on the PVN and pituitary to inhibit further release of CRH and ACTH, thus terminating stress axis activity [77]. In the hippocampus, the enzyme 11-β hydroxysteroid dehydrogenase type 1 (11βHSD1) actively converts inactive CORT to active CORT and thus provides the possibility of increasing tissue-specific CORT levels. Alterations in the levels of GR and 11βHSD1 might affect hippocampus-mediated tonic inhibition of the stress axis. This, in part, may lead to impairment in the negative feedback mechanism which, in turn results in chronic hyperactivation of the stress axis, a condition that has been implicated in the pathogenesis of metabolic syndrome [196].

Studies have reported higher urinary free cortisol [88] and post-prandial salivary cortisol [197] in obese individuals suggesting a hyperactive stress axis in obesity. Similarly, in the non-human primate model, chronic psychological stress resulted in increased body weight, visceral fat deposition, insulin resistance, glucose tolerance, enhanced cortisol secretion to ACTH stimulation and adrenal hypertrophy [86]. Considering the strong correlation between cortisol and metabolic syndrome, it is possible that prenatal stress-induced metabolic programming could be mediated through dysregulation of the stress axis in the programmed offspring. In fact, studies using prenatal stress models suggest that it results in higher corticosterone secretion at

the end of the lighting period in both males and female offspring [198], increase in CRH expression in the PVN [199] and enhanced responsiveness to stressors in the postnatal period resulting in higher circulating ACTH and cortisol levels [200]. All these changes in the HPA axis of prenatally stressed offspring have been associated with learning impairment [201, 202], increase in anxiety or depression like behavior [203, 204] and enhanced predisposition to drug abuse in the postnatal period [205]. However, their role in metabolic disorders has not been well understood.

In the current study, we hypothesized that prenatal stress in combination with postnatal HF feeding results in stress axis hyperactivation characterized by increase in NE levels in the PVN, elevated CRH levels in the ME and higher circulating CORT levels in the male DIO and DR offspring. We investigated the mRNA and protein expression of glucocorticoid receptor (GR) and 11βHSD1 in the hippocampus to assess the integrity of the negative feedback circuit in prenatally stressed DIO and DR offspring. We also hypothesize that DIO offspring would be more prone to HPA activation than DR offspring, predisposing them to a greater risk of developing obesity during the postnatal period.

Experimental Design

The study described in this chapter is designed to investigate if there is dysregulation in HPA axis function in prenatally stressed DIO and DR offspring. The same experimental protocol as described in the previous chapter was followed. Briefly, pregnant DIO (n=16) and DR (n=14) female rats were randomly divided into 3 groups each: Non-stressed Controls (DIO-NS and DR-NS), Acute prenatal Stress (DIO-AS and DR-AS) and Chronic prenatal Stress group (DIO-CS and DR-CS). Male offspring from all the treatment groups were weaned onto a chow diet (23% protein, 72% carbohydrate, and 5% calories as fat with an energy density of 3.11 kcal/g) at 3 weeks of age. At the end of 9 weeks of age, the animals in each group were sub-divided into 2 groups and were either exposed to HF diet (20% protein, 35% carbohydrate, and 45% calories as fat with an energy density of 4.73 kcal/g; Research Diets, New Brunswick, NJ) or a regular chow diet for 1 week. After 1 week of chow/HF diet treatment, all the animals were sacrificed by decapitation around noon. Serum was separated from trunk blood and stored at -80^{o}C until analyzed. Adrenal gland weight was recorded at the time of sacrifice and expressed as a ratio after normalization to the body weight of the animal. Brains were collected and immediately frozen on dry ice and later stored at -80^{o}C until further analysis. Serum was analyzed for CORT levels by RIA. Brains were sectioned (300μm thickness) and the areas that regulate stress axis, including hippocampus, PVN and median eminence (ME) were microdissected using Palkovit's microdissection technique. PVN was analyzed for NE using HPLC-EC. CRH levels were measured in the ME punches using a commercial ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA). The mRNA levels of GR and 11βHSD1 in the hippocampus were

analyzed by real time-PCR and their corresponding protein expression was analyzed through western blotting. The primers for 11βHSD1 (Ref. Seq. No. NM_017080), Glucocorticoid receptor (Ref. Seq. No. NM_012576) and β-actin (Ref. Seq. No. NM_031144) were purchased from SABiosciences, Frederick, MD. The primary antibodies used for WB are as follows: 1:500 anti-11βHSD1 rabbit polyclonal IgG (Santa Cruz Biotechnology Inc, Santa Cruz, CA), 1:500 anti-GR rabbit polyclonal IgG (Santa Cruz Biotechnology Inv, Santa Cruz, CA), 1:1000 anti-β tubulin goat polyclonal IgG (for hippocampal tissues) (Santa Cruz Biotechnology Inc, Santa Cruz, CA) and 1:1000 anti-β actin goat polyclonal IgG (for liver tissues) (Abcam, Cambridge, MA).

Results

Adrenal gland weight to body weight ratio

Adrenal gland to body weight ratio (means±SE) in prenatally stressed DIO and DR male offspring after exposure to 1 week chow or HF diet are shown separately in Fig. 4-7 and 4-8 respectively. There were no significant differences in the adrenal gland to body weight ratio between non-stressed and stressed (both acute and chronic prenatal stress) DIO and DR groups.

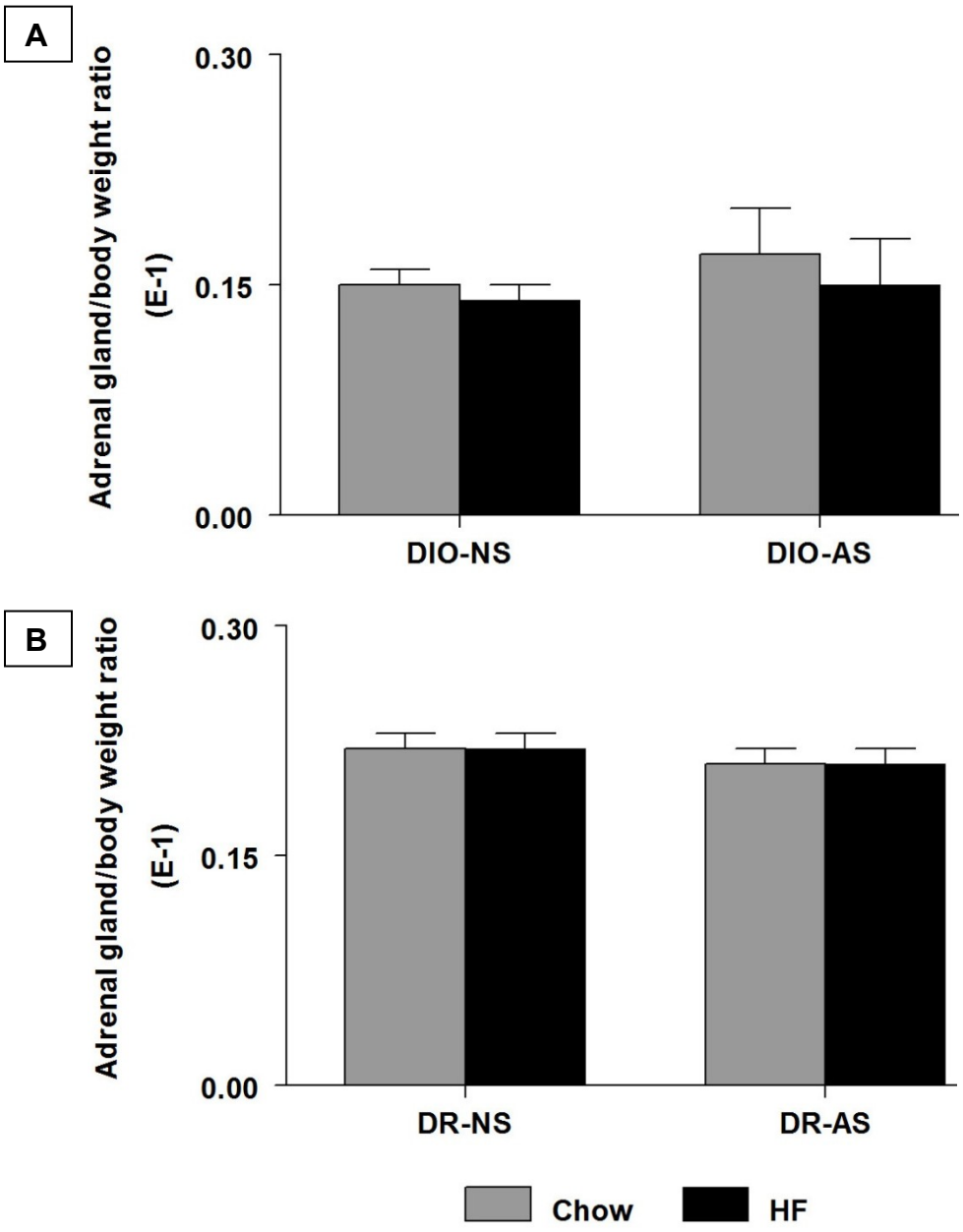

Fig 5-1: Effects of acute prenatal stress and postnatal HF diet exposure for 1 week on adrenal gland weight/body weight ratio in the DIO and DR male offspring

Adrenal gland weight/body weight ratio (means±SE) of the prenatally stressed (acute stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 5-1A and B respectively. NS-Offspring from non-stressed dams, AS- Offspring from dams subjected to acute stress during pregnancy. No significant difference was observed between the groups.

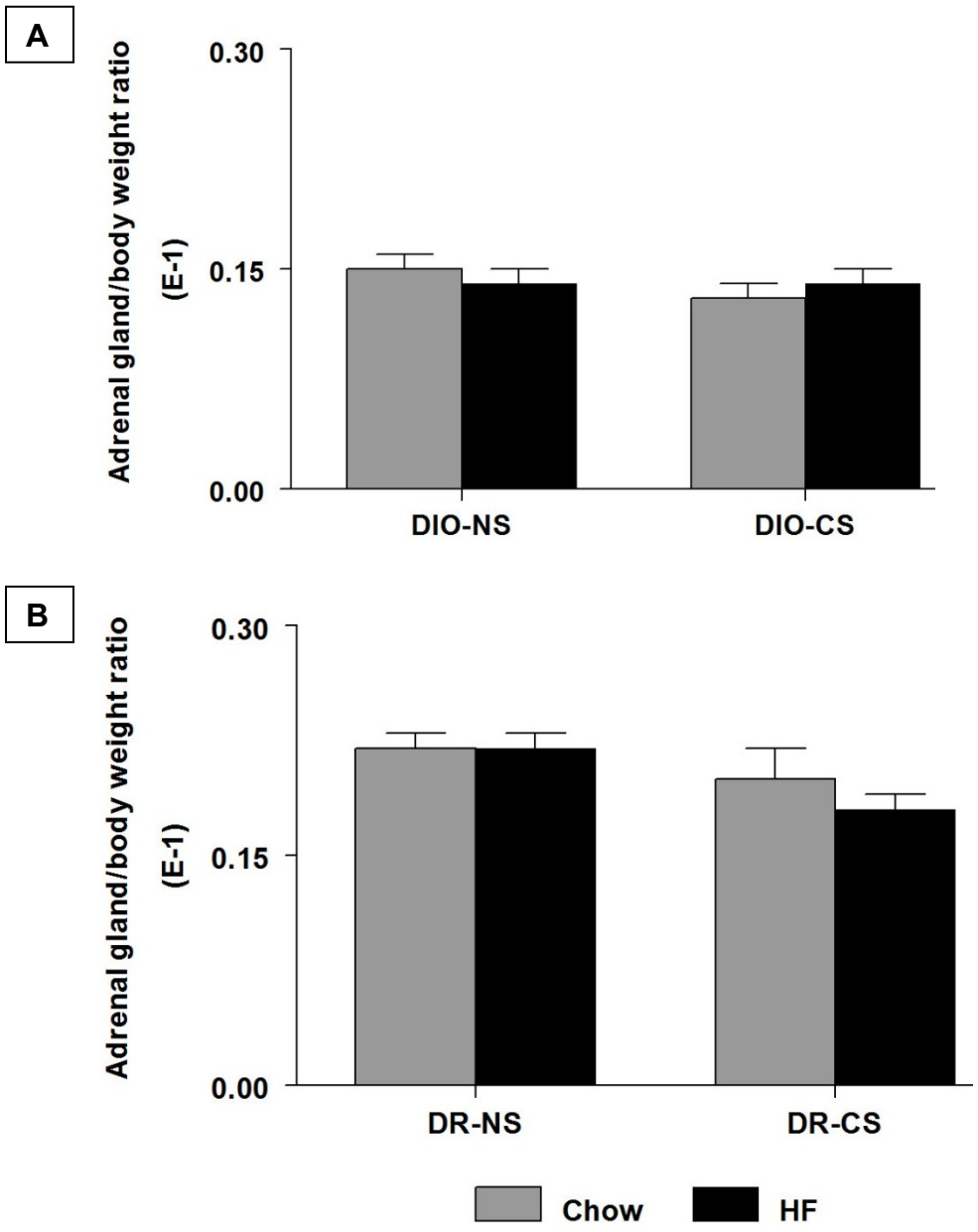

Fig 5-2: Effects of chronic prenatal stress and postnatal HF diet exposure for 1 week on adrenal gland weight/body weight ratio in the DIO and DR male offspring

Adrenal gland weight/body weight ratio (means±SE) of the prenatally stressed (chronic stress) and non-stressed DIO and DR male offspring after 1 week chow or HF diet exposure are shown in Fig. 5-2 A and B respectively. NS-Offspring from non-stressed dams, CS-Offspring from dams who underwent chronic restraint stress during pregnancy. No significant difference was observed between the groups.

NE levels in the PVN of the hypothalamus

Changes in NE levels (means±SE, pg/μg of protein) in the PVN of DIO and DR offspring subjected to acute or chronic prenatal stress are shown in Fig. 5-3 and 4 respectively. In both DIO and DR non-stressed animals, 1 week HF diet exposure did not alter NE levels in the PVN. However, 1 week HF diet exposure significantly increased NE levels in the PVN in the acute stress group when compared to its chow-fed counterparts in both DIO (62.1±11.0 vs 33.8±4.1, $p<0.05$) and DR offspring (66.7±6.3 vs 48.7±3.6, $p<0.05$) (Fig. 5-3A). Further in the DR animals, NE levels in the PVN of the HF fed acute stress offspring (66.7±6.3,$p<0.05$) was significantly greater than the non-stressed controls (32.2±4.2 and 37.6±2.4 in chow and HF fed groups) (Fig. 5-3B). Two-way ANOVA revealed a significant effect of acute stress in increasing NE levels in DR animals but not in DIOs.

With respect to chronic prenatal stress, NE levels in the PVN were significantly higher in the HF-fed DIO group (98.7±10.7, $p<0.05$) when compared to its chow (57.3±7.5) and also chow (49.6±3.2) and HF-fed (56.3±4.5) non-stressed counterparts (DIO-NS) (Fig. 5-4A). In contrast, in DR animals, both chow-fed and HF-fed DR-CS animals (58.9±6.0 and 64.1±4.4 in chow and HF groups respectively) had significantly higher NE levels in the PVN compared to the non-stressed groups (32.2±4.2 and 37.6±2.4 in chow and HF fed groups) (Fig. 5-4B). We observed an overall effect of chronic stress but no effect of diet in increasing NE levels in the PVN of DR animals. On the other hand, there was a significant effect of diet, stress and stress x diet interaction in the increase in NE levels observed in the DIO animals.

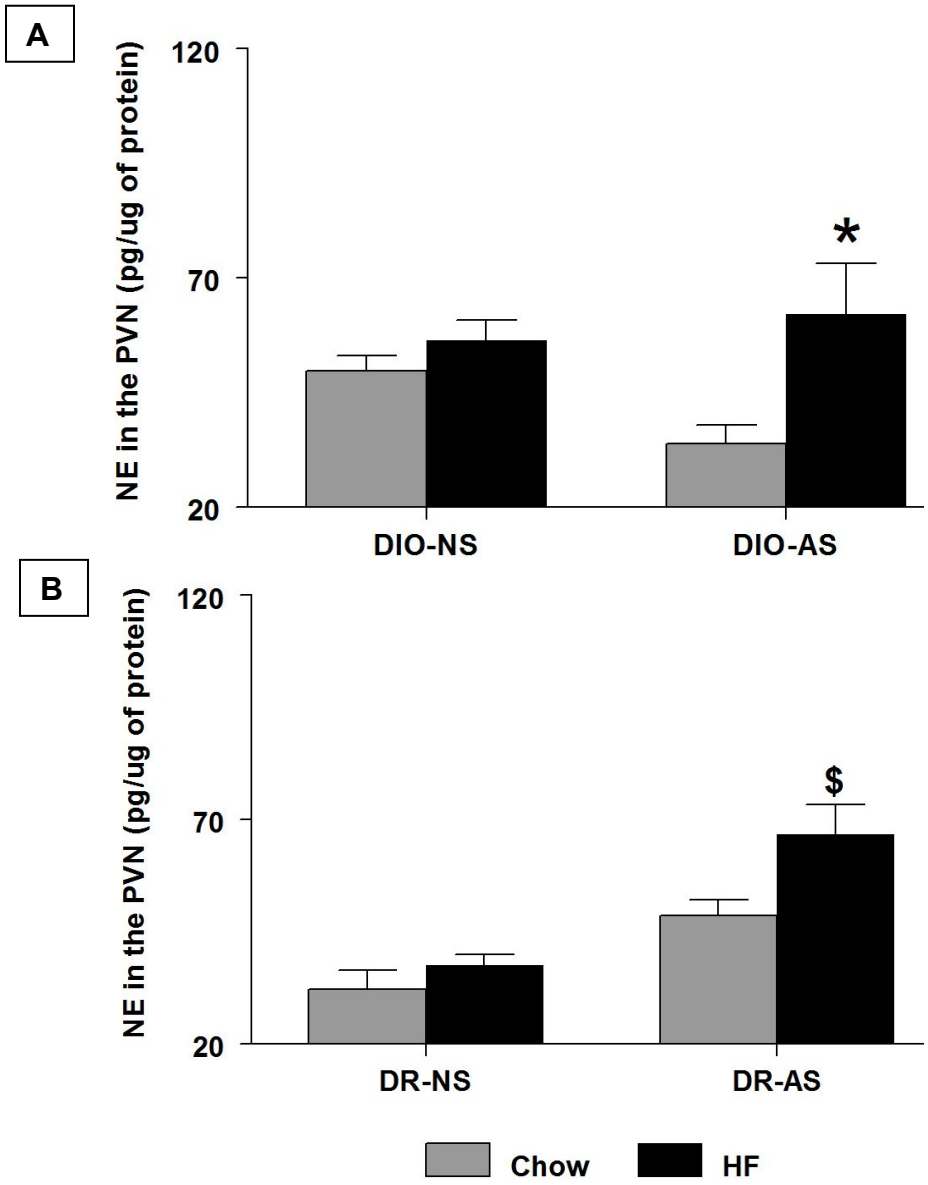

Fig 5-3: Effects of acute prenatal stress on NE levels in the PVN of the DIO and DR treatment groups

NE levels in the PVN across various treatment groups in the DIO and DR offspring are shown in Fig.5-3A and B respectively. NS-Offspring from non-stressed dams, AS-Offspring from dams subjected to acute stress during pregnancy. '*' denotes significant ($p<0.05$) difference from their respective chow groups and '$' denotes difference from all the other groups.

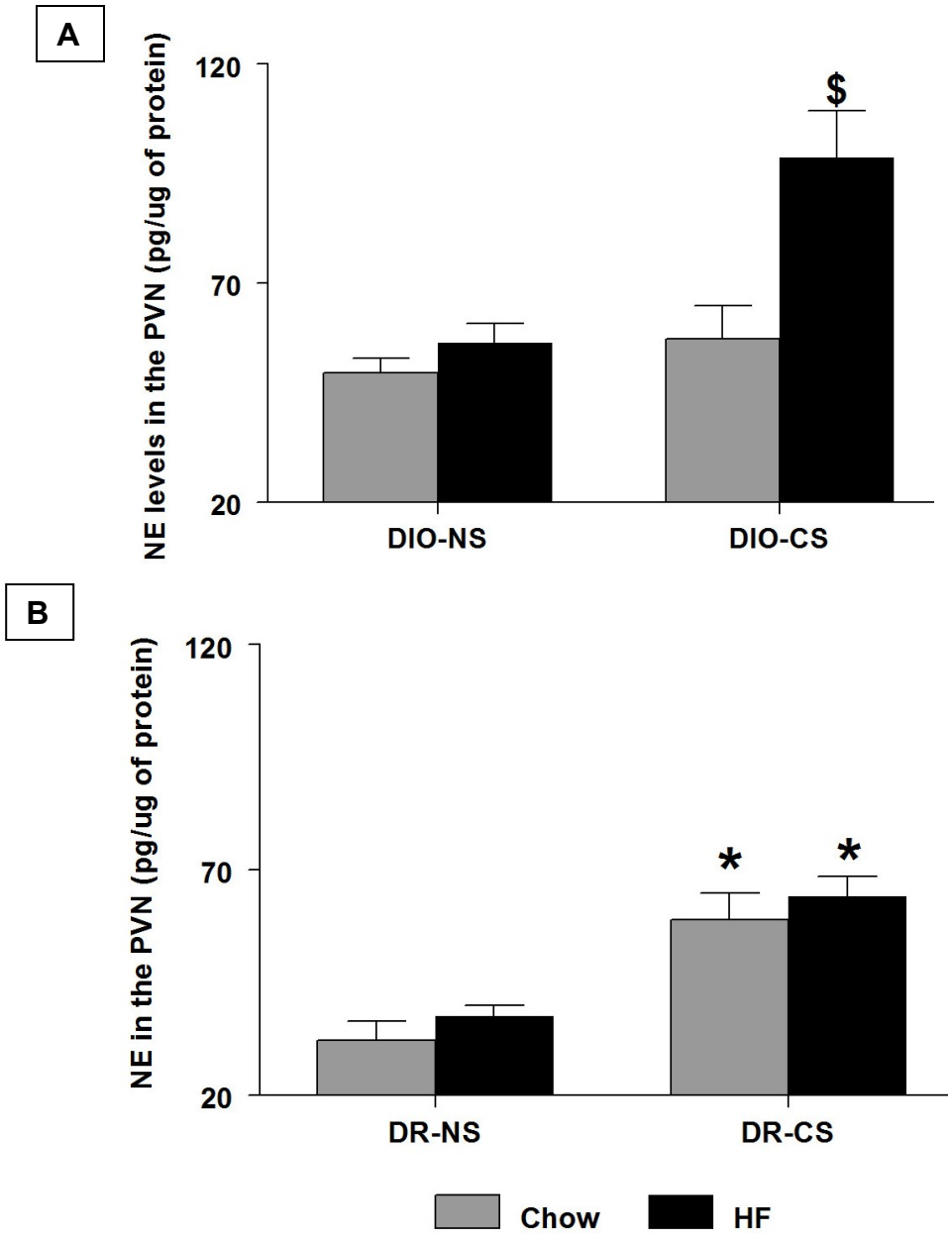

Fig 5-4: Effects of chronic prenatal stress on NE levels in the PVN of the DIO and DR treatment groups

NE levels in the PVN across various treatment groups in the DIO and DR offspring are shown in Fig.5-4A and B respectively. NS-Offspring from non-stressed dams, CS-Offspring from dams subjected to chronic restraint stress during pregnancy. '*' denotes significant ($p<0.05$) difference from chow and HF fed non-stressed groups. '$' denotes significant difference from all the groups

CRH levels in the median eminence (ME)

CRH levels (means±SE, pg/µg of protein) in the ME of DIO and DR rats after acute and chronic prenatal stress are shown in Fig. 5-5 and 6 respectively. There was a significant increase in CRH levels in the acute stress offspring both on chow (108.9±11.9) and HF (111.6±11.5) compared to the DIO-NS chow group (59.9±8.6). On the other hand, in DR animals, CRH levels in the ME were significantly ($p<0.05$) higher in the acute stress groups (126.3±10.4 and 164.9±14.1 in chow and HF-fed groups) when compared to the non-stressed controls (70.5±8.9 chow fed groups) (Fig. 5-5B). Also the increase in CRH observed in DR-CS HF was significantly greater than in the DR-NS HF (94.04±6.5). Two-way ANOVA revealed a significant effect of acute stress in increasing CRH levels in both DR and DIO animals.

Similar to acute stress, chronic stress also produced a significant increase in CRH levels in the ME in both DIO and DR animals. Irrespective of the diet, DIO-CS animals (152.3±27.2 and 208.6±29.7 in chow and HF groups respectively, $p<0.05$) had significantly higher CRH levels in the ME compared to the DIO-NS groups (59.9±8.6 and 95.5±7.5 in chow and HF fed groups respectively) (Fig. 5-6A). Similarly, CRH levels in the ME were significantly higher in the DR-CS group (201.02±24.6 and 240.7±21.4 in chow and HF groups respectively) compared to the non-stressed controls (70.5±8.9 and 94.04±6.5 in chow and HF fed groups) (Fig. 5-6B). We observed an overall effect of chronic stress but not diet in increasing CRH levels in the ME in both DIO and DR animals.

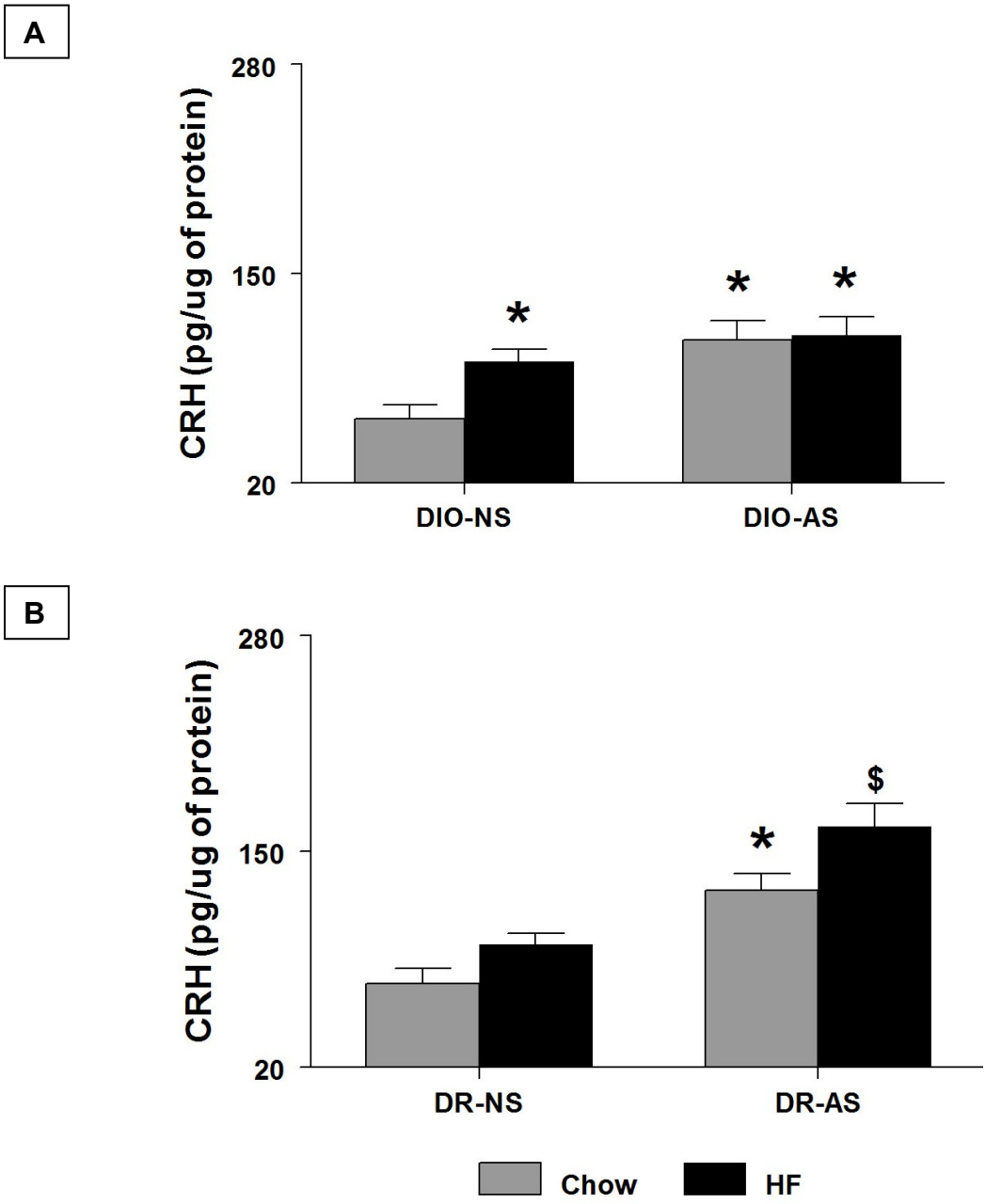

Fig 5-5: Effects of acute prenatal stress on CRH levels in the ME of DIO and DR offspring

CRH levels in the ME across various treatment groups in the DIO and DR offspring are shown in Fig.5-5A and B respectively. NS-Offspring from non-stressed dams, AS-Offspring from dams subjected to acute stress during pregnancy. '*' denotes significant ($p<0.05$) difference from chow fed non-stressed groups. '$' denotes significant difference from all the groups.

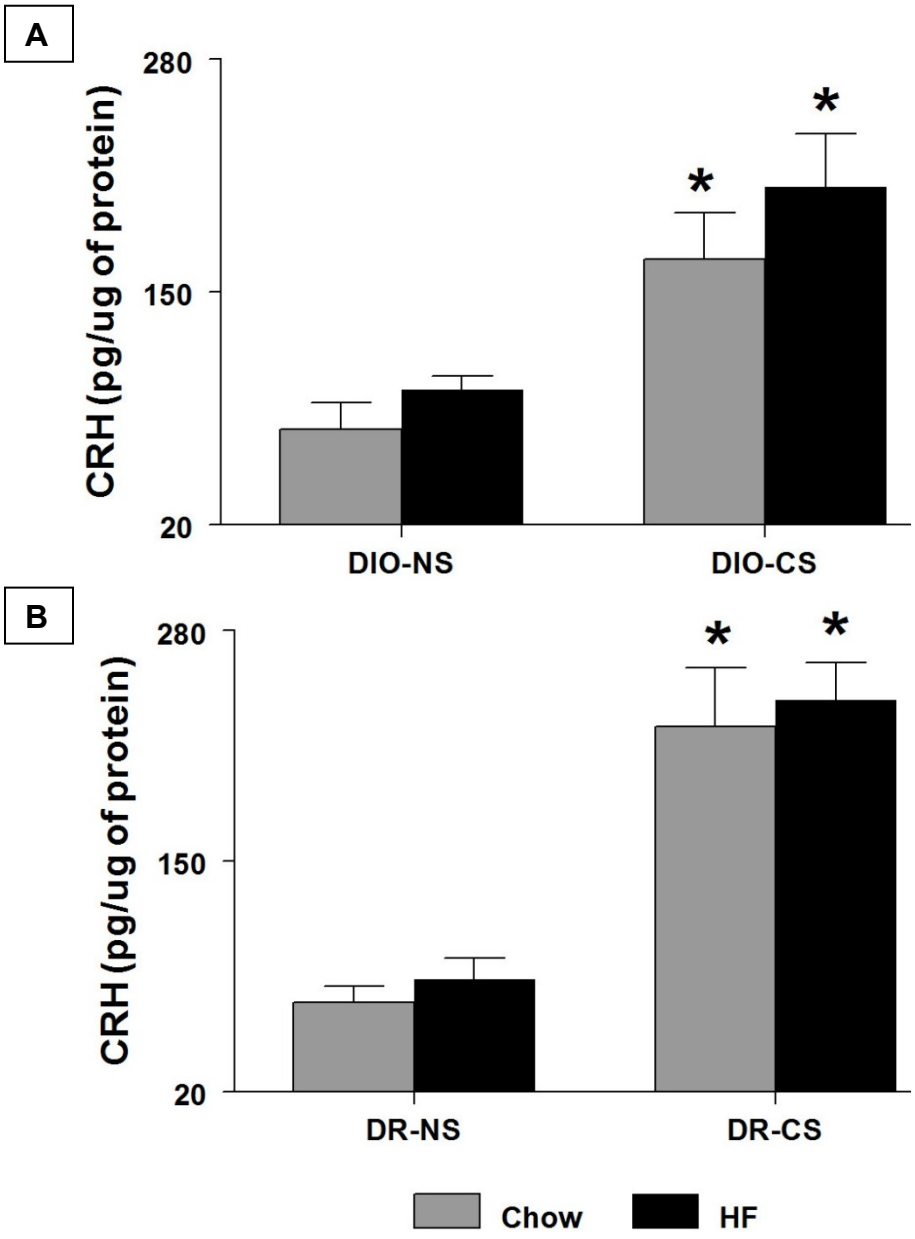

Fig 5-6: Effects of chronic prenatal stress on CRH levels in the ME of the DIO and DR offspring

CRH levels in the ME across various treatment groups in the DIO and DR offspring are shown in Fig.5-6A and B respectively. NS-Offspring from non-stressed dams, CS-Offspring from dams subjected to chronic restraint stress during pregnancy. '*' denotes significant ($p<0.05$) difference from chow and HF fed non-stressed groups.

Serum Corticosterone

Serum Corticosterone (means±SE, ng/ml) levels measured at the time of sacrifice after 1 week chow/HF diet exposure are shown in Fig. 5-7 and 5-8. Unlike NE and CRH levels, acute and chronic prenatal stress did not alter serum CORT levels in both DIO and DR animals. HF diet exposure for 1 week also did not have any effect on CORT levels in both DIO and DR animals.

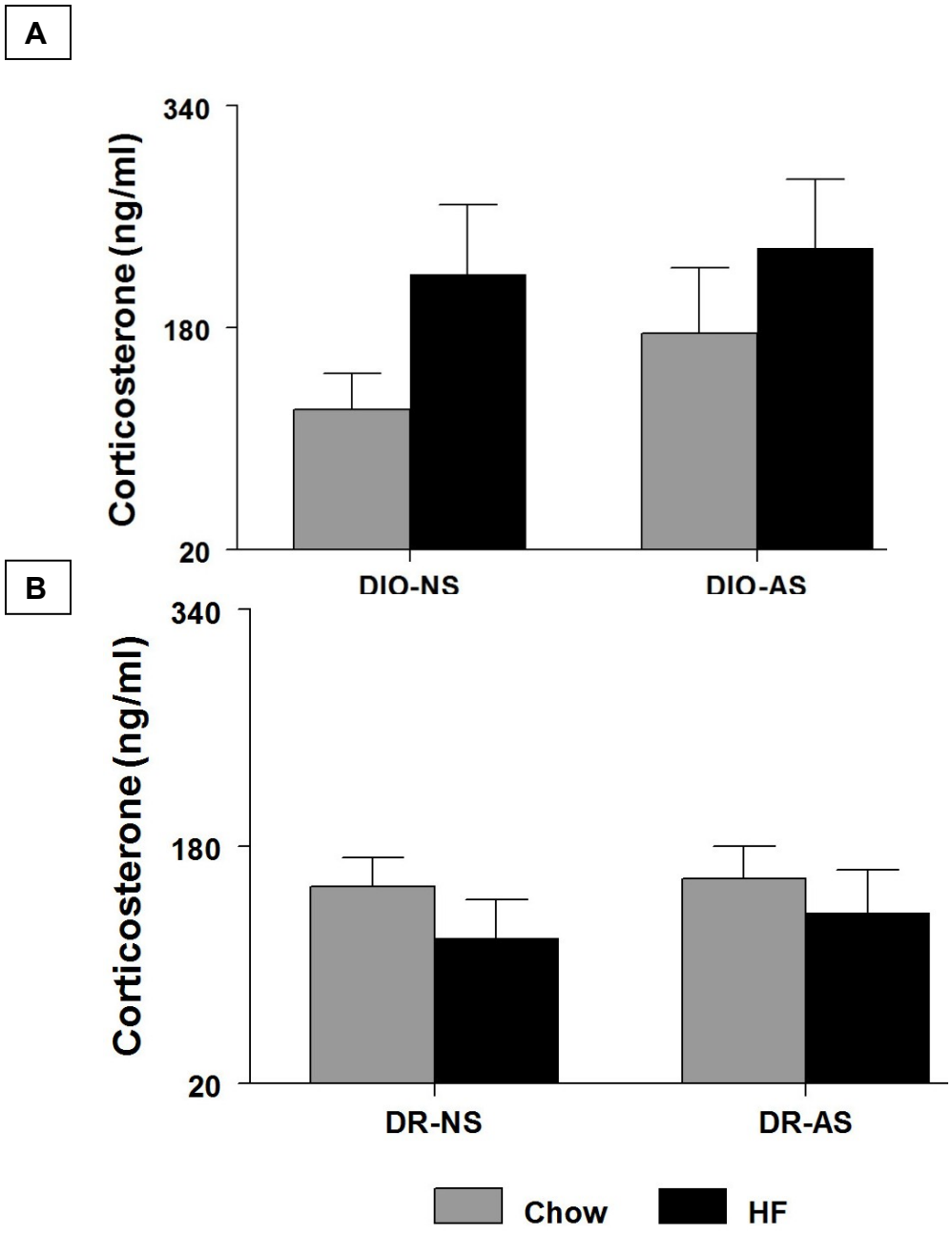

Fig. 5-7 A and B: Effects of acute prenatal stress on serum CORT levels in DIO and DR male offspring

Serum CORT levels (means±SE, ng/ml) in DIO and DR animals across different treatment groups are shown in Fig. 5-7 A and B respectively. NS-Offspring from non-stressed dams, AS- Offspring from dams subjected to acute stress during pregnancy. No statistically significant differences in serum CORT levels were observed between the groups.

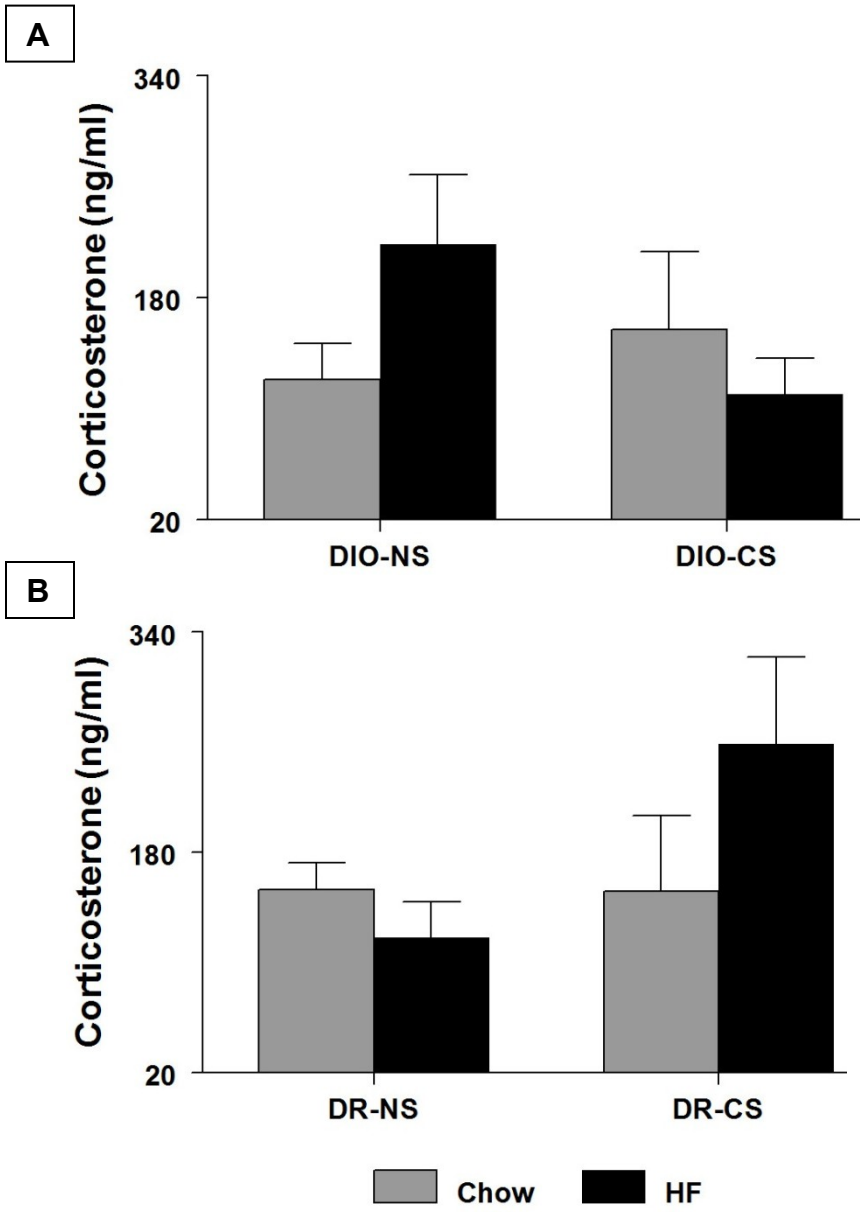

Fig. 5-8 A and B: Effects of chronic prenatal stress on serum CORT levels in DIO and DR male offspring

Serum CORT levels (means±SE, ng/ml) in DIO and DR animals across different treatment groups are shown in Fig. 5-8 A and B respectively. NS-Offspring from non-stressed dams, CS- Offspring from dams subjected to chronic restraint stress during pregnancy. No statistically significant differences in serum CORT levels were observed between the groups.

GR mRNA and protein expression in the hippocampus

GR mRNA values were expressed as GR mRNA: β-actin ratio in the hippocampus of the DIO and DR offspring in Fig. 5-9 A and B respectively. There were no differences in the gene expression of GR in the hippocampus in both prenatally stressed DIO and DR offspring when compared with their non-stressed counterparts. Exposure to 1 week HF diet also did not produce any changes in hippocampal GR expression.

The protein levels of GR in the hippocampus were expressed as a ratio of GR to β-tubulin in the DIO and DR offspring in Fig. 5-9 C and D respectively. Acute and chronic prenatal stress did not have any effect on the protein levels of GR in the hippocampus in both DIO and DR offspring.

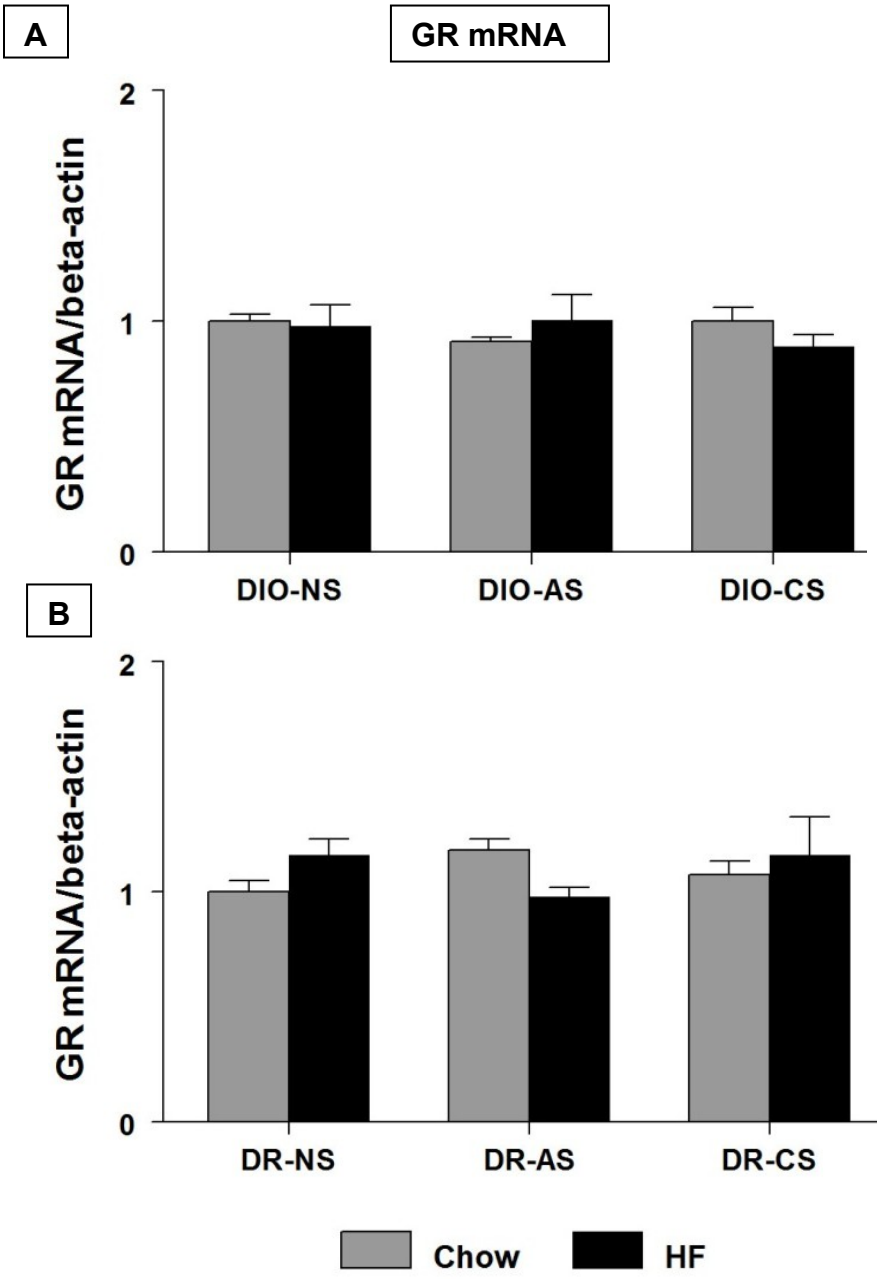

Fig. 5-9 A-B: Effects of acute and chronic prenatal stress on mRNA expression of GR in the hippocampus

The mRNA levels of GR across different treatment groups in the DIO and DR offspring are shown in Fig. 5-9 A and B respectively. NS-Offspring from non-stressed dams, AS- Offspring from dams subjected to acute stress during pregnancy and CS-Offspring from dams subjected to chronic restraint stress during pregnancy.

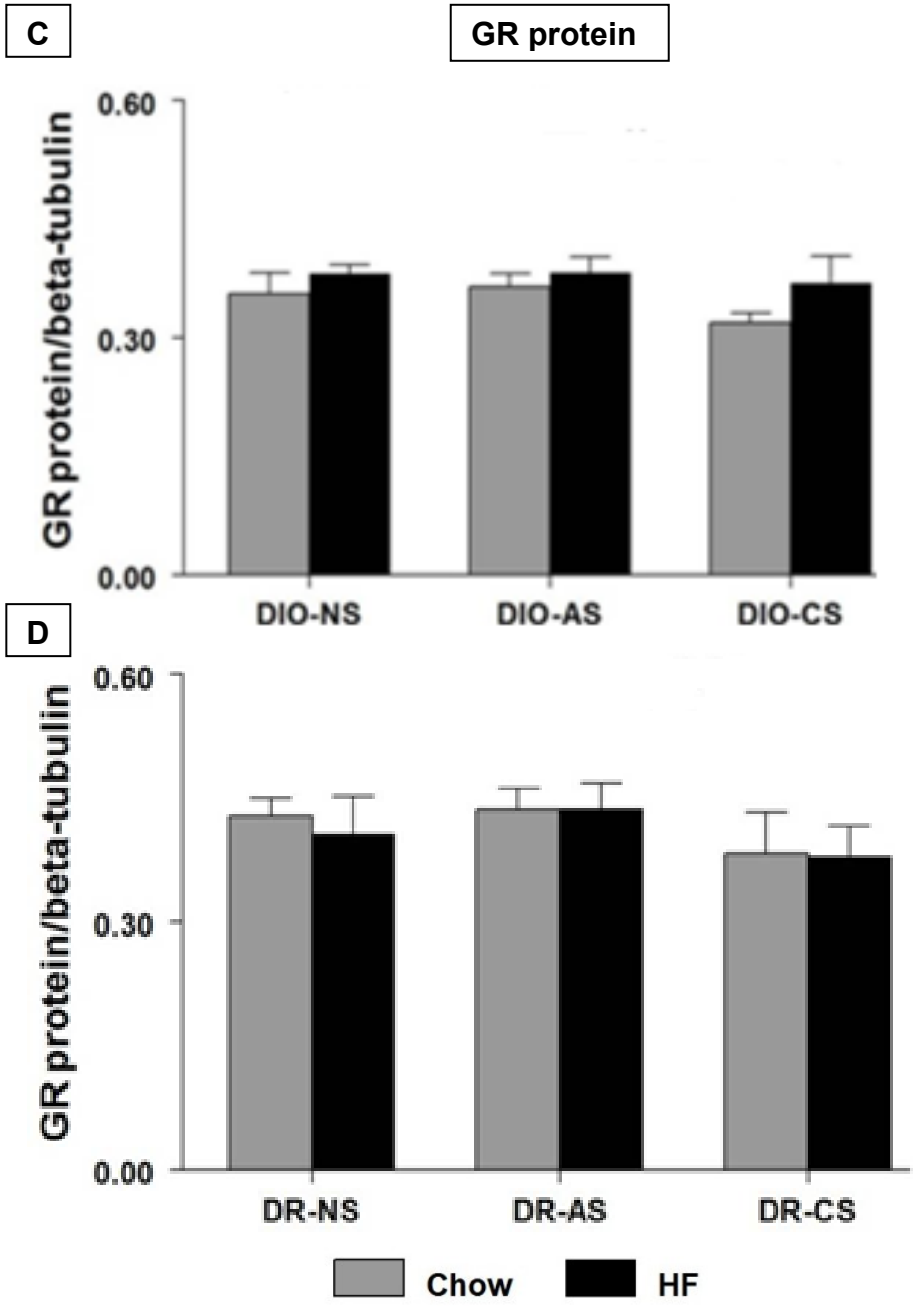

Fig. 5-9 C-D: Effects of acute and chronic prenatal stress on protein expression of GR in the hippocampus

The protein levels of GR across different treatment groups in the DIO and DR offspring are shown in Fig. 5-9 C and D respectively. NS-Offspring from non-stressed dams, AS- Offspring from dams subjected to acute stress during pregnancy and CS-Offspring from dams subjected to chronic restraint stress during pregnancy.

11βHSD1 mRNA and protein expression in the hippocampus

11βHSD1 mRNA values were expressed as 11βHSD1 mRNA: β-actin ratio in the hippocampus of the DIO and DR offspring and depicted in Fig. 5-10 A and B respectively. Prenatal stress did not have any effect on the gene expression of 11βHSD1 in the hippocampus in both prenatally stressed DIO and DR offspring when compared with their non-stressed counterparts. Exposure to 1 week HF diet also did not produce any changes in the hippocampal GR expression.

The protein levels of 11βHSD1 in the hippocampus were normalized to β-tubulin and expressed as a ratio of 11βHSD1 to β-tubulin in the DIO and DR offspring in Fig. 5-10 C and D respectively. There were no statistically significant differences in the 11βHSD1 protein levels in the hippocampus in both DIO and DR prenatally stressed offspring.

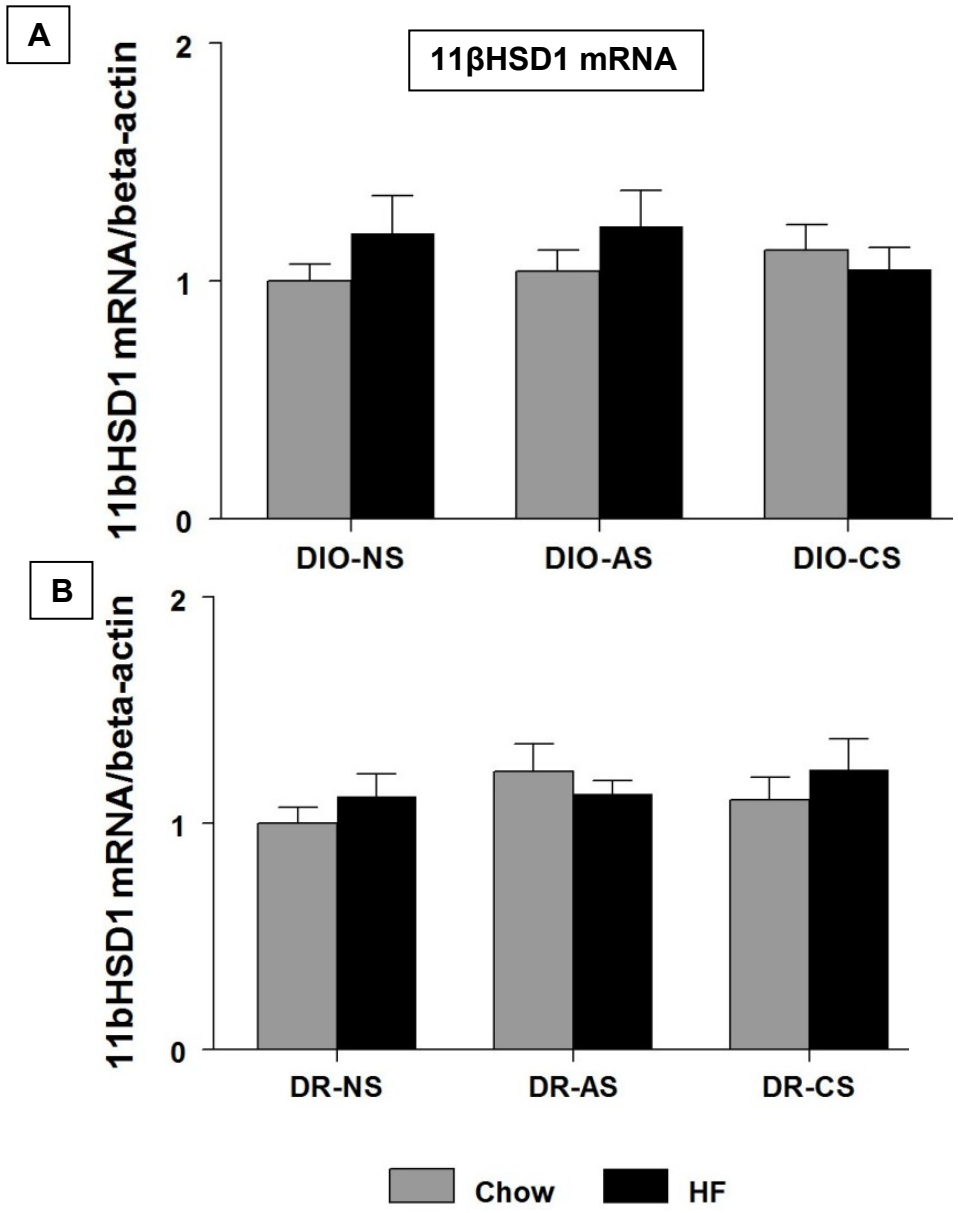

Fig. 5-10 A-B: Effects of acute and chronic prenatal stress on mRNA expression of 11βHSD1 in the hippocampus

The mRNA levels of 11βHSD1 across different treatment groups in the DIO and DR offspring are shown in Fig. 5-10 A and B respectively. NS-Offspring from non-stressed dams, AS- Offspring from dams subjected to acute stress during pregnancy and CS-Offspring from dams subjected to chronic restraint stress during pregnancy.

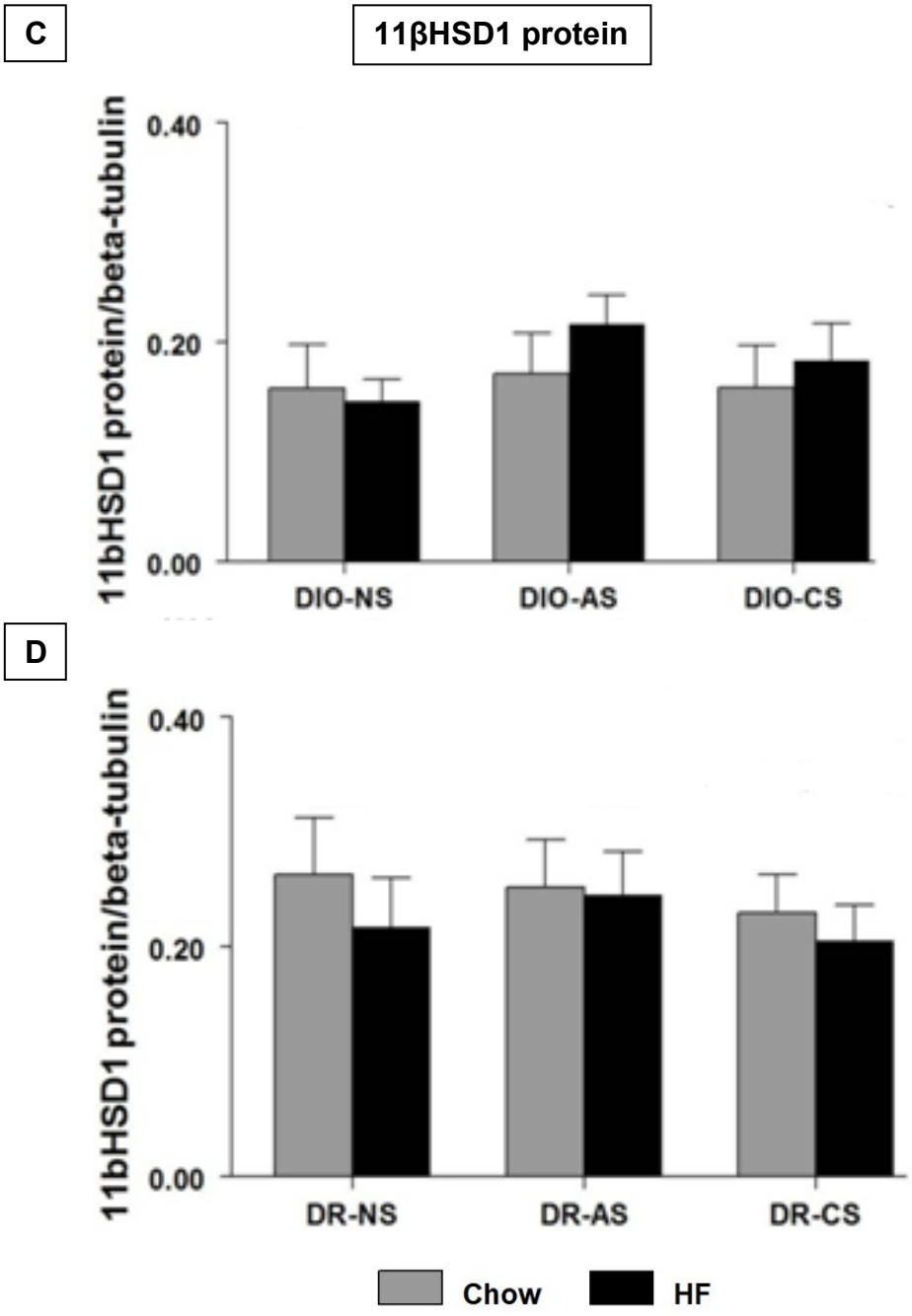

Fig. 5-10 C-D: Effects of acute and chronic prenatal stress on protein expression of 11βHSD1 in the hippocampus

The protein levels of 11βHSD1 across different treatment groups in the DIO and DR offspring are shown in Fig. 5-9 C and D respectively. NS-Offspring from non-stressed dams, AS- Offspring from dams subjected to acute stress during pregnancy and CS-Offspring from dams subjected to chronic restraint stress during pregnancy.

Discussion

The association between stress axis hyperactivity and obesity/metabolic syndrome is well documented in the literature. Cushing's disease characterized by increase in circulating glucocorticoids levels features abdominal obesity suggesting a link between glucocorticoids and obesity [193, 194]. Glucocorticoids promote obesity and insulin resistance through multiple ways. Centrally, glucocorticoids increase feed intake and body weight gain by acting on the feeding centers [206]. Peripherally, glucocorticoids act in the liver and the adipose tissue to facilitate gluconeogenesis [207] and adipocyte differentiation [208]. In light of all these findings, we wanted to investigate if prenatal stress would increase stress axis activity and lead to adverse metabolic outcomes in the DIO as compared to the DR offspring.

In the DIO offspring, acute prenatal stress did not affect NE levels in the PVN but significantly increased CRH levels in the median eminence. On the other hand, chronic prenatal stress significantly increased NE and CRH levels in the PVN and median eminence in the DIO offspring as compared to the non-stressed offspring. HF diet exposure for 1 week did not have any additive effect to chronic stress induced increases in NE and CRH levels in the DIO animals. To our surprise, both acute and chronic prenatal stress elicited an increase in NE and CRH levels in the DR offspring which did not show any metabolic alterations unlike DIO animals. Furthermore, in both the DIO and DR offspring, increases in NE and CRH levels were not accompanied by a corresponding increase in CORT levels. The findings from this study demonstrate dysregulation of stress axis in the prenatally stressed offspring.

Our results are in agreement with other studies examining the effects of prenatal stress on stress axis function in terms of NE and CRH concentrations in the offspring. Prenatal stress has been reported to increase NE concentrations in the hypothalamus [209] and increase NE turnover rate in the brain stem suggesting higher NE release [210]. Also, CRH concentrations in the amygdala, which is another stress regulating center in the brain, was increased in the prenatally stressed offspring [211]. Apart from being a part of stress axis, PVN also regulates feeding behavior. Acute and chronic infusion of NE in the PVN produces hyperphagia and promotes obesity [212, 213]. Hence, the increase in PVN NE concentrations observed in the prenatally stressed offspring would explain the increase in calorie intake observed in the DIO and DR offspring.

One of the mechanisms behind the increase in NE and CRH observed in prenatally stressed offspring is impairment in the negative feedback regulation of stress axis. Previously, studies have reported decrease in hippocampal GR expression in the prenatally stressed offspring [214-216]. In order to test if a similar phenomenon exists in DIO/DR model, we examined the mRNA and protein expression of GR and 11βHSD1 in the hippocampus in the offspring. Our results showed that prenatal stress did not produce any changes in the hippocampal GR and 11βHSD1 expression in both DIO and DR offspring. As an alternate mechanism, other molecules like free fatty acids (FFA) or pro-inflammatory cytokines can also activate HPA axis by increasing NE activity in the PVN [217]. However, it is not clear if prenatal stress *per se* increases the levels of circulating FFA and/or cytokines.

In the current study, despite increases in NE and CRH levels, we did not observe any changes in baseline CORT levels between stressed and non-stressed DIO and DR offspring. Previously, studies have demonstrated a similar finding where there was no difference in baseline CORT levels in prenatally stressed offspring on postnatal day (PND) 21 [169, 215] and PND90 [215]. On the other hand, few other studies have also observed an increase in baseline CORT in the offspring subjected to prenatal stress [33] and prolonged CORT response to stress [214]. The disparity in the findings might be due to the differences in the stress protocol and the age at which CORT measurements were made in the offspring. Further, based on the results from the present study, it is still not clear whether increased renal clearance could have masked the true CORT levels in the prenatally stressed DIO/DR offspring. Additional studies on CORT metabolite measurements in the urine are needed to answer this question.

Despite no detectable changes in circulating CORT levels, glucocorticoids might still play a role in the peripheral metabolic outcomes observed in the prenatally stressed DIO offspring through the enzyme, 11βHSD1. 11βHSD1 is involved in pre-receptor metabolism of CORT in the metabolically active peripheral tissues like the liver and the adipose tissue where it converts inactive 11-dehydroCORT to active CORT [92]. Irrespective of circulating CORT levels, increase in 11βHSD1 expression in these tissues will result in amplification of glucocorticoid actions leading to obesity and insulin resistance [92]. Hence, in the next chapter we investigated the role of 11βHSD1 in the liver and the adipose tissue in mediating prenatal stress induced metabolic alterations in the DIO and DR male offspring.

The results from the present study indicate that prenatal stress produces significant changes in the HPA axis of both DIO and DR offspring. Whether these changes in the HPA axis mediate prenatal stress-induced metabolic alterations is still not clear. Also, the current study only assessed basal HPA axis function. Further studies on stress-induced alterations in HPA axis function are needed to completely understand the functioning of stress axis in the prenatally stressed offspring.

CHAPTER 6

EFFECTS OF PRENATAL STRESS ON 11βHSD1 EXPRESSION IN THE LIVER AND THE ADIPOSE TISSUE

Introduction

Literature supports a strong relationship between cortisol and obesity. However, the circulating cortisol levels are not elevated in some obese subjects [99, 100], suggesting that altered cortisol metabolism or increased tissue sensitivity to circulating cortisol may play a role in the development of obesity in these subjects. Glucocorticoid's actions in the peripheral tissues are not only dependent on its circulating levels, but also on the availability of glucocorticoid receptors and the activity of the enzyme, 11-β hydroxysteroid dehydrogenase type 1 (11βHSD1) [92].

11βHSD1 is a NADPH dependent microsomal enzyme encoded by the gene HSD11B1 [95]. It is highly expressed in metabolically active tissues like liver, adipose tissue and also in the central nervous system [93]. *In vivo*, 11βHSD1 acts as a reductase and converts inactive 11-dehydrocorticosterone to CORT in rodents or inactive cortisone to cortisol in humans. Despite low circulating CORT, the intracellular levels of CORT available for binding with GR may be increased by the activity of 11βHSD1 in the tissues. Hence, 11βHSD1 is involved in tissue-specific amplification of glucocorticoid actions and has been implicated in the pathogenesis of obesity and insulin resistance. A polymorphism in intron 3 of the gene encoding 11βHSD1 results in obesity and insulin resistance in children further emphasizing its role in metabolic disorders [218].

In humans and obese animal models, there is an increase in the expression and activity of 11βHSD1 in the liver and adipose tissue [219-223]. Overexpression of 11βHSD1 in the adipose tissue mimicked metabolic syndrome featuring insulin-resistant diabetes, hyperlipidemia with increased corticosterone content in the adipose tissue

[102]. On the other hand, over expression of 11βHSD1 in the liver alone results in a milder form of metabolic syndrome with insulin resistance and hypertension without hyperglycemia and obesity [224]. Conversely, inhibition of 11βHSD1 in rodents confers protection against diet-induced obesity and atherosclerosis [103] [104]. All these studies underline the importance of the role of 11βHSD1 in obesity and insulin resistance.

Studies have suggested that prenatal factors could program 11βHSD1 expression in the offspring. Dexamethasone injection during late gestation resulted in persistent elevations in 11βHSD1 expression and activity in the liver and adipose tissue [225]. Similarly, maternal nutrient restriction during pregnancy and gestational diabetes also increased the expression of GR and 11βHSD1 in the adipose tissue [226, 227]. Considering the fact that, CORT levels are unaltered in the prenatally stressed offspring, it is possible that metabolic syndrome associated with prenatal stress in the DIO offspring might be mediated through GR and 11βHSD1 pathway in the liver and the adipose tissue. In the present study, we hypothesized that prenatal stress increases the expression of 11βHSD1 in the liver and adipose tissue and results in amplification of glucocorticoid's actions.

Experimental Design

The same experimental protocol as described in chapter 4 was followed in this study. After 1 week chow or HF diet treatment, the animals were sacrificed by decapitation and liver and visceral adipose tissue (VAT) was collected from the animals. The tissues were divided into 2 portions. One portion was snap-frozen on dry ice and stored at -80^{0}C until further analysis. The second portion was fixed in 10% neutral buffered formalin for 3 days and transferred to 70% ethanol prior to being embedded in paraffin blocks, and sectioned (5μm). In the adipose tissue, H and E stained sections were used for adipocyte area measurements. GR and 11βHSD1 mRNA expression was analyzed using real time-PCR and their corresponding protein levels were estimated using western blotting and immunohistochemistry.

Results

Adipocyte area

Adipocyte area (means±SE, μm^2) measured in all the DIO and DR groups are shown in Fig.6-1 and 6-2 respectively. Chow fed DIO-AS animals (6194.6±656.5, $p<0.05$) had significantly higher adipocyte area compared to its non-stressed counterpart (4519.6±403.2). Exposure to 1 week HF diet significantly increased the adipocyte area in all the DIO groups when compared to their chow-fed counterparts: DIO-CS (9154.4±332.4 vs 6163.2±192.9, $p<0.05$), DIO-AS (11033.5±374.3 vs 6194.6±656.5, $p<0.05$) and DIO-NS (8639.1±426.3 vs 4519.6±403.2, $p<0.05$). Chow fed DIO-CS and DIO-AS animals had significantly ($p<0.05$) higher adipocyte area compared to its non-stressed counterpart. Also, the increase in the adipocyte area in the DIO-AS animals after HF diet exposure was significantly greater compared to the HF-fed DIO-NS. Two-way ANOVA revealed a significant acute stress and chronic stress effect ($p<0.05$) and diet effect ($p<0.05$) in increasing adipocyte area in the DIO offspring.

We did not observe any differences in the adipocyte area between the DR groups. No stress or diet effect was observed in the DR animals with respect to adipocyte area.

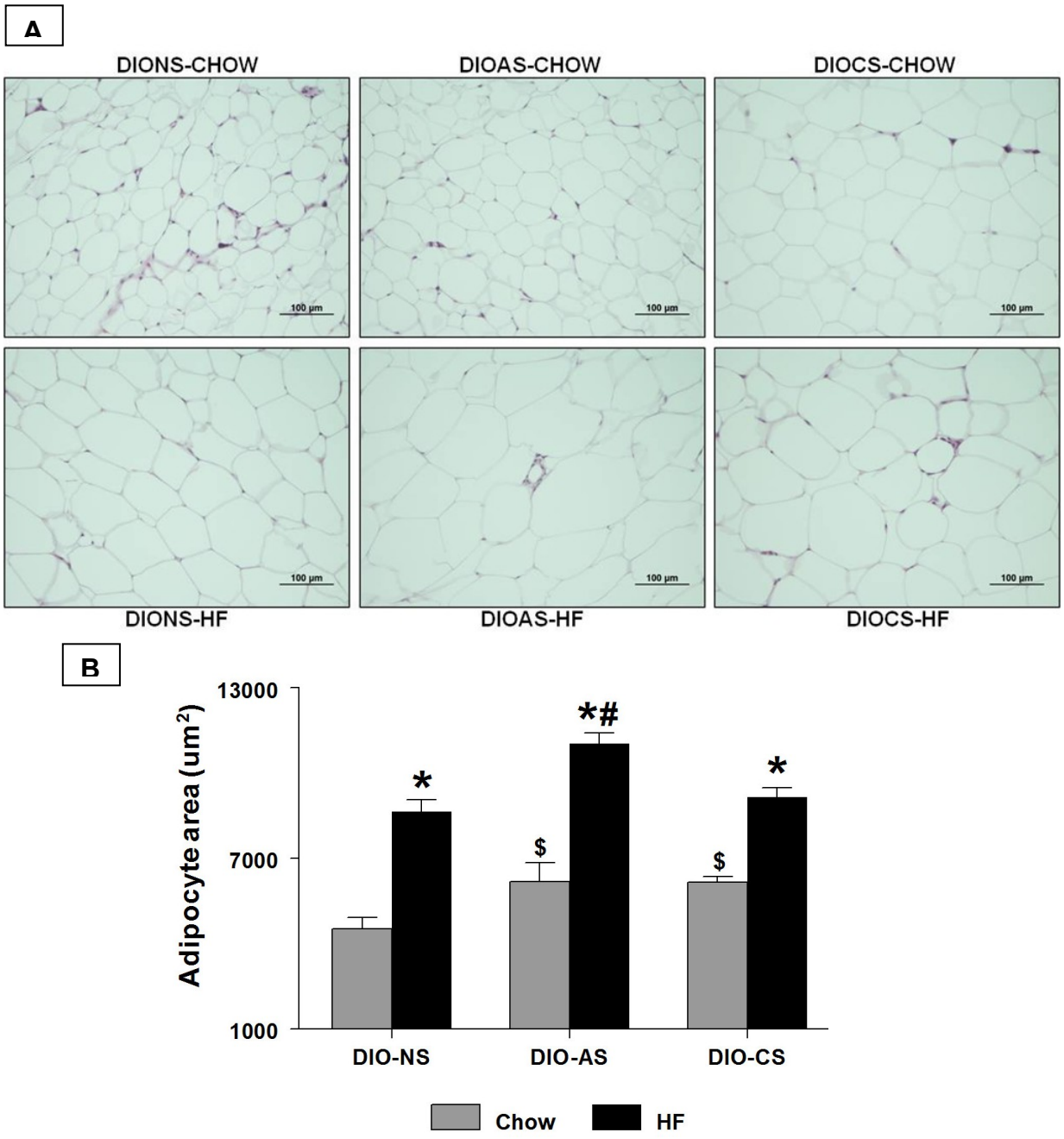

Fig. 6-1 A and B: Effects of prenatal stress (acute and chronic) and postnatal HF diet on the adipocyte area in DIO offspring

Representative histological sections of visceral adipose tissue (20X magnification) from all the DIO groups are shown in Fig. 6-1A. The average adipocyte area measurements (means±SE, μm^2) measured in the histological sections from different treatment groups are shown in Fig.6-1B. '*' denotes significant difference from their respective chow groups. '$' denotes significant difference from the DIO-NS chow group. '#' denotes significant difference from DIO-NS HF group.

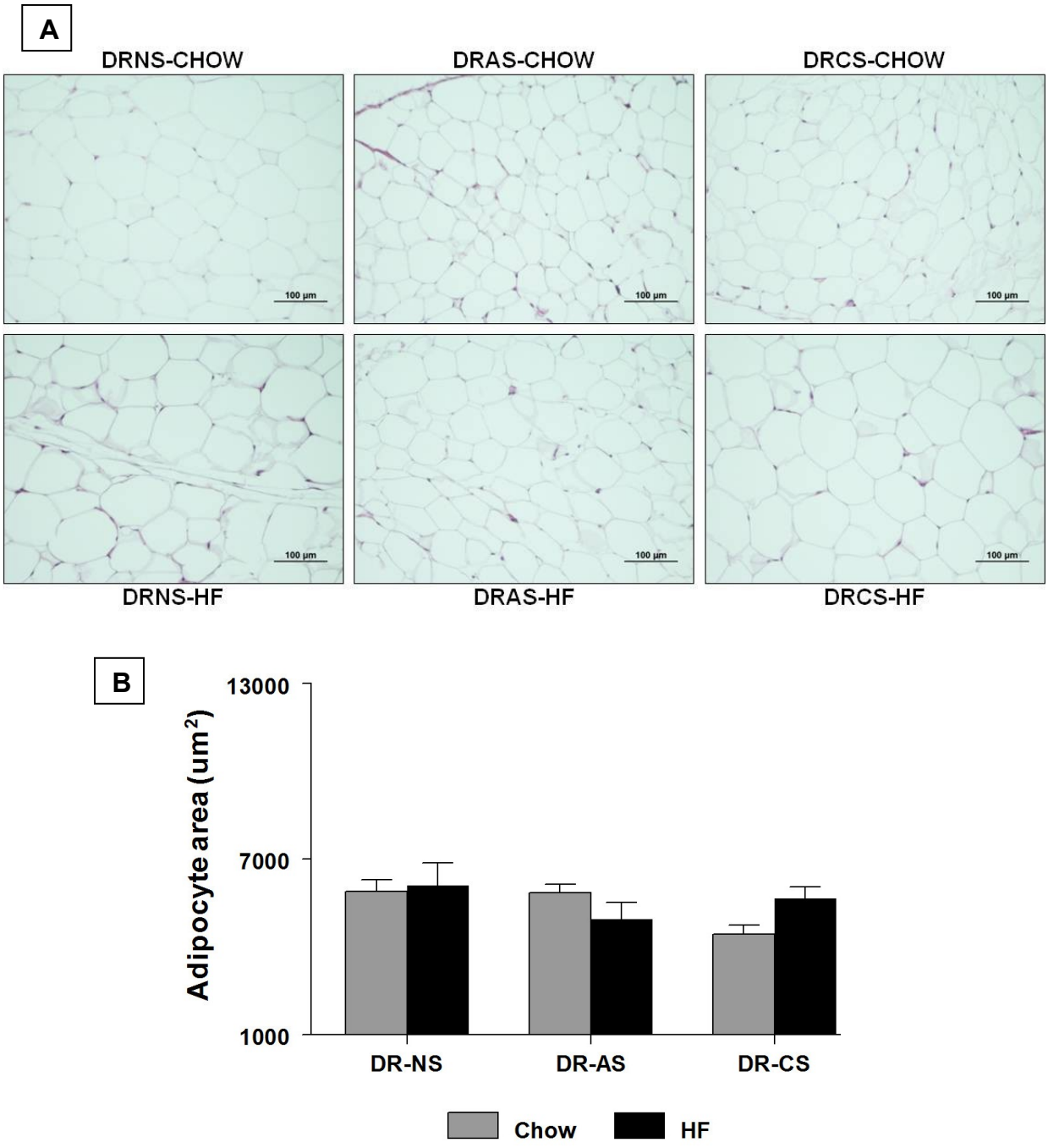

Fig. 6-2 A and B: Effects of prenatal stress (acute and chronic) and postnatal HF diet on the adipocyte area in DIO offspring

Representative histological sections of visceral adipose tissue (20X magnification) from all the DIO groups are shown in Fig. 6-2A. The average adipocyte area measurements (means±SE, μm^2) measured in the histological sections from different treatment groups are shown in Fig.6-2B. Note there were no differences in the adipocyte area between the DR groups.

11βHSD1 mRNA and protein levels in the liver

11βHSD1 mRNA values were expressed as 11βHSD1 mRNA: β-actin ratio in the hippocampus of the DIO and DR offspring and depicted in Fig. 6-3 A and B respectively. HF diet exposure for 1 week produced a significant ($p<0.05$) down-regulation in 11βHSD1 mRNA expression in the liver in all the DIO and DR groups compared to their respective chow-fed counterparts. Prenatal stress did not have any effect on 11βHSD1 mRNA expression in the liver in the DIO and DR groups. Two-way ANOVA analysis showed a significant diet bit no prenatal stress effect in 11βHSD1 mRNA expression in the liver in both the DIO and DR groups.

The protein levels of 11βHSD1 in the DIO and DR groups are shown in Fig. 6-3 C and D respectively. No differences in the protein levels of 11βHSD1 were observed between non-stressed and stressed DIO and DR groups.

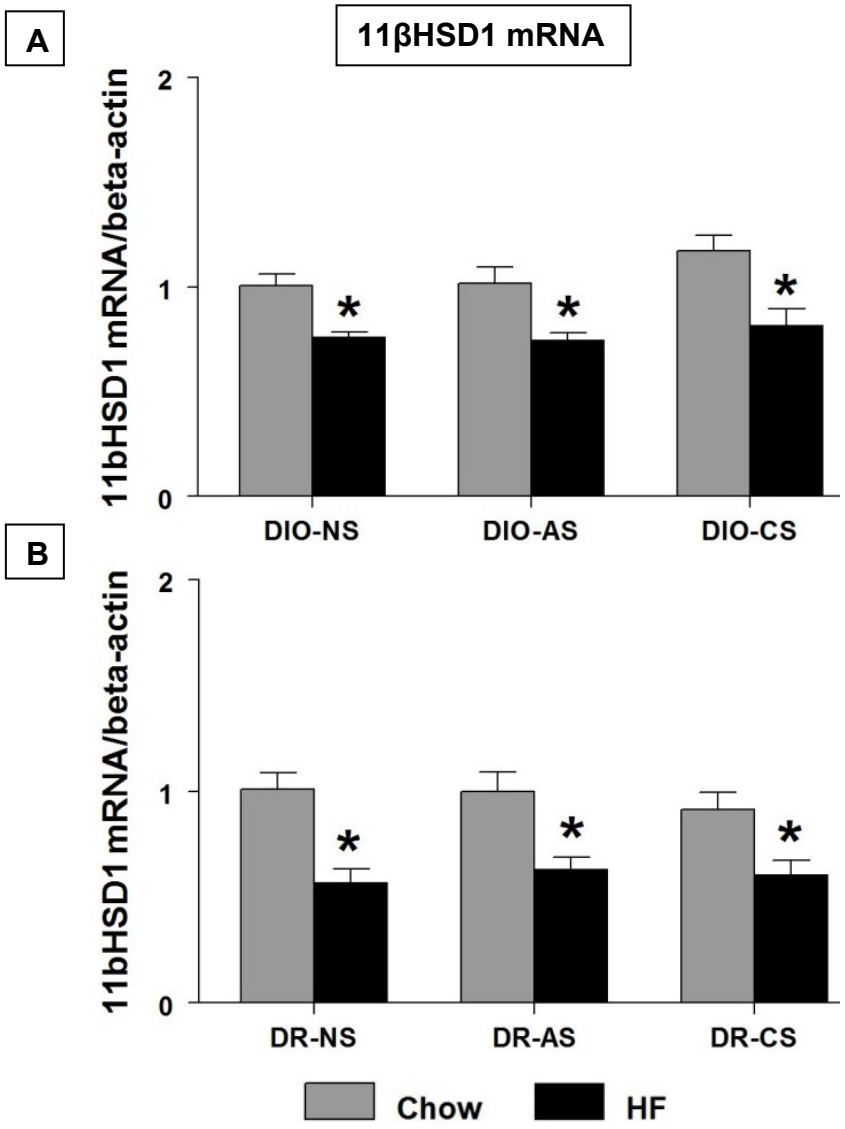

Fig. 6-3 A and B: Effects of prenatal stress and postnatal HF diet exposure on 11βHSD1 mRNA levels in the liver of the DIO and DR offspring

The mRNA levels of 11βHSD1 (fold change normalized to beta-actin) in the liver of DIO and DR treatment groups (n=5/group) are shown in Fig. 6-3A and B respectively. Note there was a significant diet effect in all the groups. But no differences were observed between non-stressed and stressed DIO and DR groups. '*' denotes significant difference ($p<0.05$) from their respective chow fed groups.

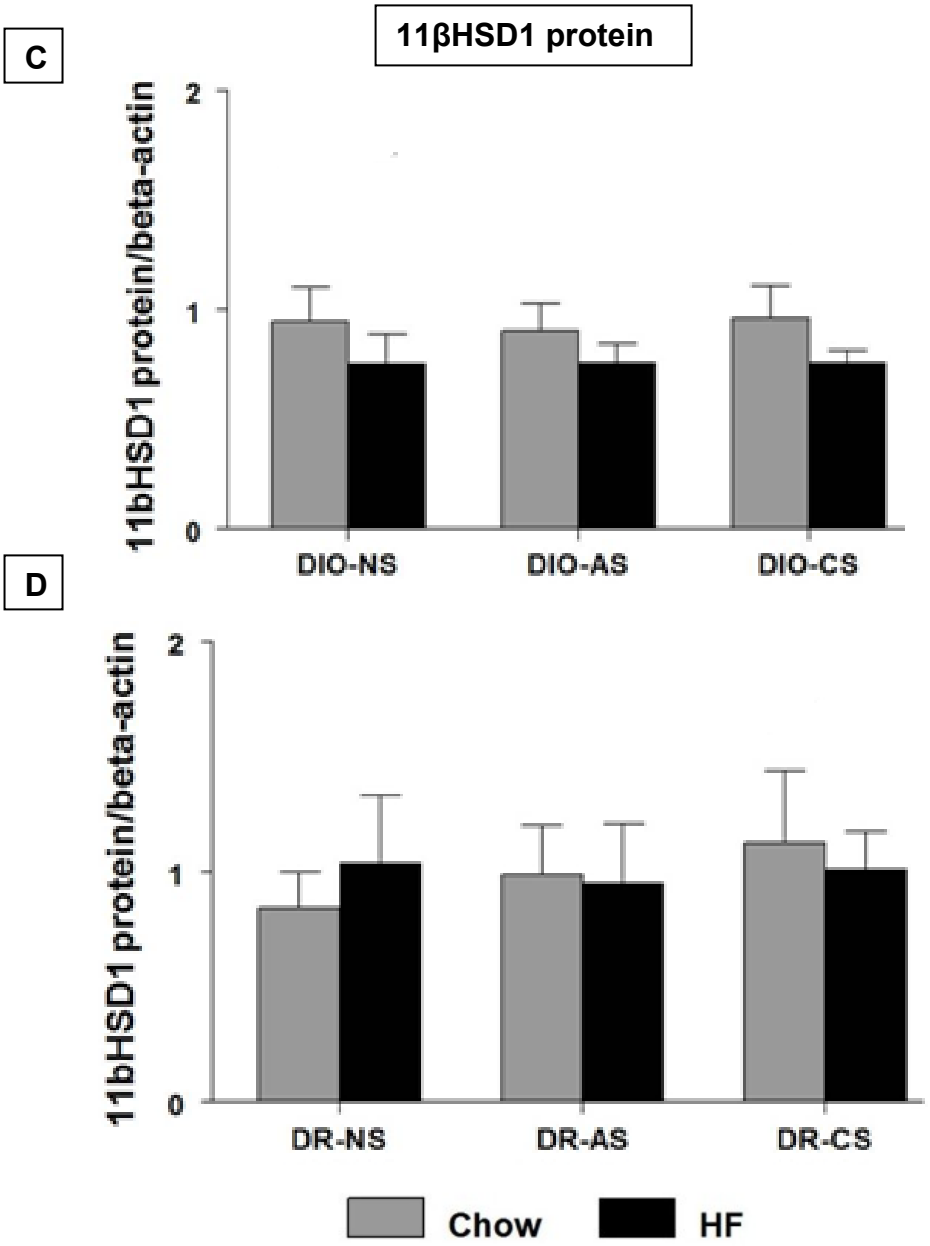

Fig. 6-3 C and D: Effects of prenatal stress and postnatal HF diet exposure on 11βHSD1 protein levels in the liver of the DIO and DR offspring

The protein levels of 11βHSD1 in the liver of DIO and DR groups (n=5/group) are shown in Fig. 6-3 C and D respectively. No differences in the protein levels were observed between the groups.

GR mRNA expression in the liver

GR mRNA levels in the liver of DIO and DR offspring were normalized to the house keeping gene, β-actin and expressed as a ratio of GR mRNA/β-actin in Fig. 6-4A and B respectively. On chow diet, no differences in GR expression were observed between stressed and non-stressed DIO groups. On high fat diet, Prenatal acute stress resulted in a moderate increase (fold change, 1.66±0.13 $p<0.05$) in the GR expression in the liver of the DIO offspring compared to DIO-NS offspring (1.0±0.05). Prenatal chronic stress did not affect the expression of GR in the liver of the DIO offspring (Fig.6-4A). Two-way ANOVA showed a significant acute stress effect but no diet effect in increasing GR expression in the liver of the DIO offspring. In the DR offspring, prenatal stress (both acute and chronic) and postnatal HF diet did not produce any changes in the GR expression in the liver (Fig.6-4B).

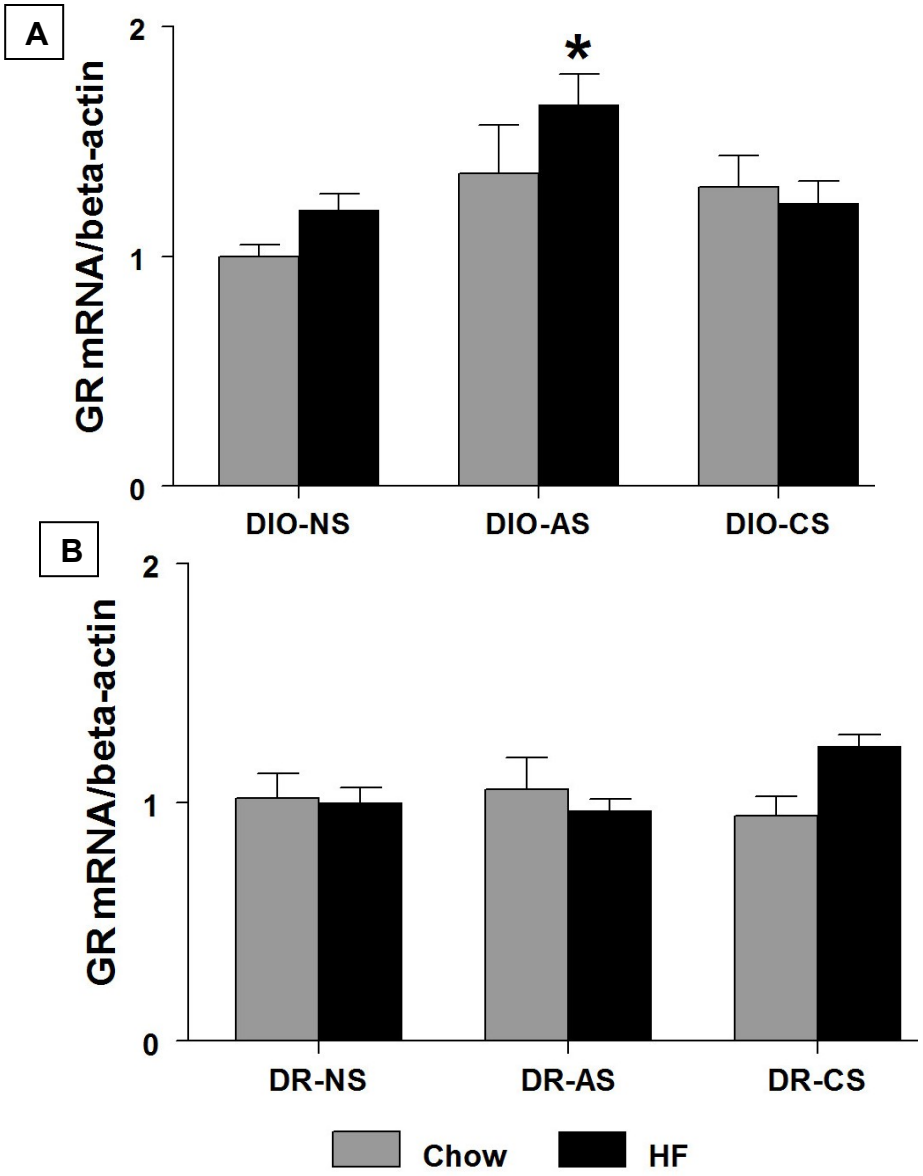

Fig. 6-4A and B: Effects of prenatal stress and postnatal HF diet exposure on GR mRNA levels in the liver of the DIO and DR offspring

The mRNA levels of GR (fold change normalized to beta-actin) in the liver of DIO and DR treatment groups (n=5/group) are shown in Fig. 6-4A and B respectively. '*' denotes significant difference ($p<0.05$) from NS groups. A significant acute stress effect was observed in the increase noted in the GR expression in the liver of the DIO offspring.

11βHSD1 and GR mRNA expression in the visceral adipose tissue (VAT)

The mRNA levels of 11βHSD1 and GR in the prenatally stressed offspring are shown in Fig. 6-5A-B and C-D respectively. On chow diet, prenatal chronic stress produced a moderate increase (1.3±0.1 fold change, $p<0.05$) in the mRNA expression of 11βHSD1 in the VAT of DIO offspring when compared to its non-stressed counterparts (0.99±0.02). No differences 11βHSD1 expression was observed between the acute stress group and non-stressed group on chow diet. Exposure to 1 week HF diet produced a significant down-regulation in the mRNA expression of 11βHSD1 in the chronic stress DIO offspring. In the DR offspring, prenatal stress did not produce any changes in the 11βHSD1 expression in the VAT.

With respect to GR expression, prenatal stress did not produce any changes both in the DIO and DR offspring except that HF diet exposure significantly reduced GR expression in the DR-CS offspring.

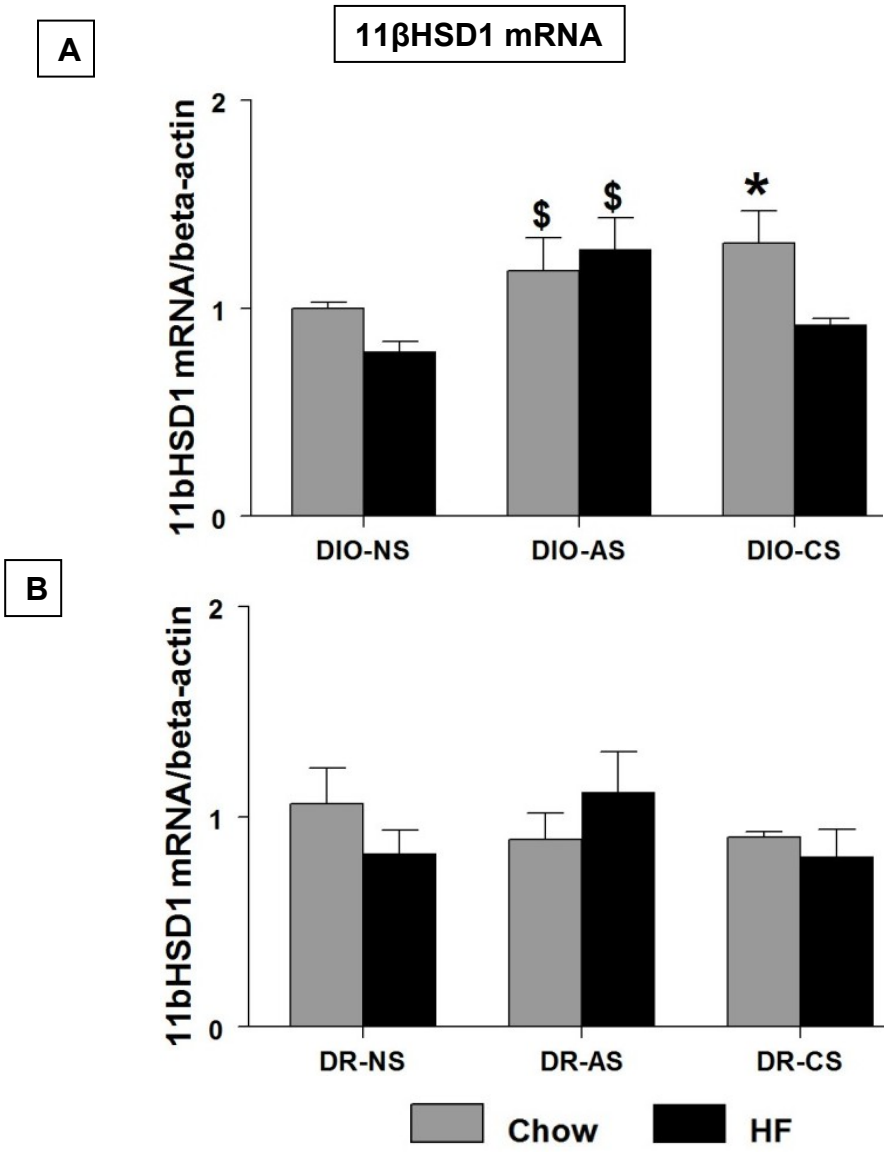

Fig. 6-5 A-B: Effects of acute and chronic prenatal stress on mRNA expression of 11βHSD1 in the visceral adipose tissue

The mRNA levels of 11βHSD1 across different treatment groups in the DIO and DR offspring are shown in Fig. 6-5 A and B respectively. NS-Offspring from non-stressed dams, AS- Offspring from dams subjected to acute stress during pregnancy and CS-Offspring from dams subjected to chronic restraint stress during pregnancy. '*' denotes significant difference from NS groups and CS-HF group. '$' denotes significant difference from NS-HF group and '#' denotes significant difference from all chow groups.

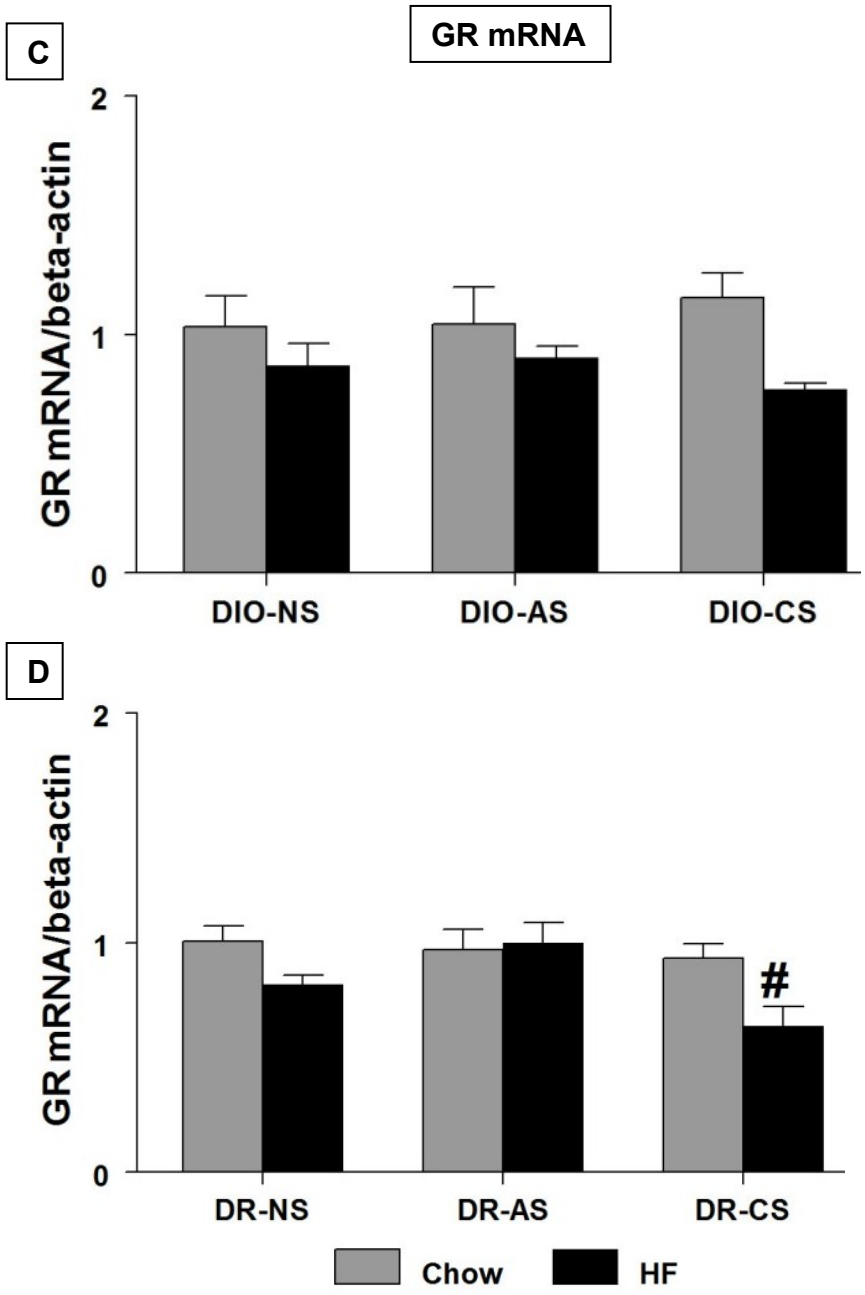

Fig. 6-5 C-D: Effects of acute and chronic prenatal stress on mRNA expression of GR in the visceral adipose tissue

The mRNA levels of GR in different treatment groups in the DIO and DR offspring are shown in Fig. 6-5 C and D respectively. NS-Offspring from non-stressed dams, AS-Offspring from dams subjected to acute stress during pregnancy and CS-Offspring from dams subjected to chronic restraint stress during pregnancy. '$' denotes significant difference from NS-HF group and '#' denotes significant difference from all chow groups.

11βHSD1 protein levels in the VAT

11βHSD1 protein levels in the VAT were expressed as the percentage of adipocytes positive for 11βHSD1 staining per field in the DIO and DR offspring and depicted in Fig. 6-6 A and B respectively. Representative images for 11βHSD1 staining in the VAT are shown in Fig. 6-7. In the DIO offspring on chow diet, there was a significant increase in 11βHSD1 expression in the chronic stress group (31.9±4.3, $p<0.05$) but not in the acute stress group (18.2±2.9) when compared with the non-stressed group (20.01±1.5). On the other hand, 1 week HF diet exposure significantly increased 11βHSD1 expression in the VAT in both chronic stress (42.3±6.7) and acute stress group (30.3±2.9) when compared with the chow (20.01±1.5) and HF-fed (16.01±2.3) non-stressed groups (Fig-6-6A).

In the DR chronic stress offspring, both chow (43.6±2.5, $p<0.05$) and HF diet (48.3±2.8, $p<0.05$) groups had greater 11βHSD1 expression in the VAT when compared with the chow (4.7±0.5) and HF fed (20.4±2.5) non-stressed offspring. Also in the acute stress offspring, there was a significant increase in VAT 11βHSD1 expression in both the chow (12.72±1.67, $p<0.05$) and HF diet (14.8±3.3, $p<0.05$) groups when compared to the DR-NS chow group (4.7±0.5) (Fig-6-6B). HF diet exposure for 1 week increased 11βHSD1 expression in the VAT only in the non-stressed group and not in the acute stress and chronic stress offspring. In both DIO and DR offspring, we observed a significant acute and chronic stress effect in increasing 11βHSD1 expression in the VAT.

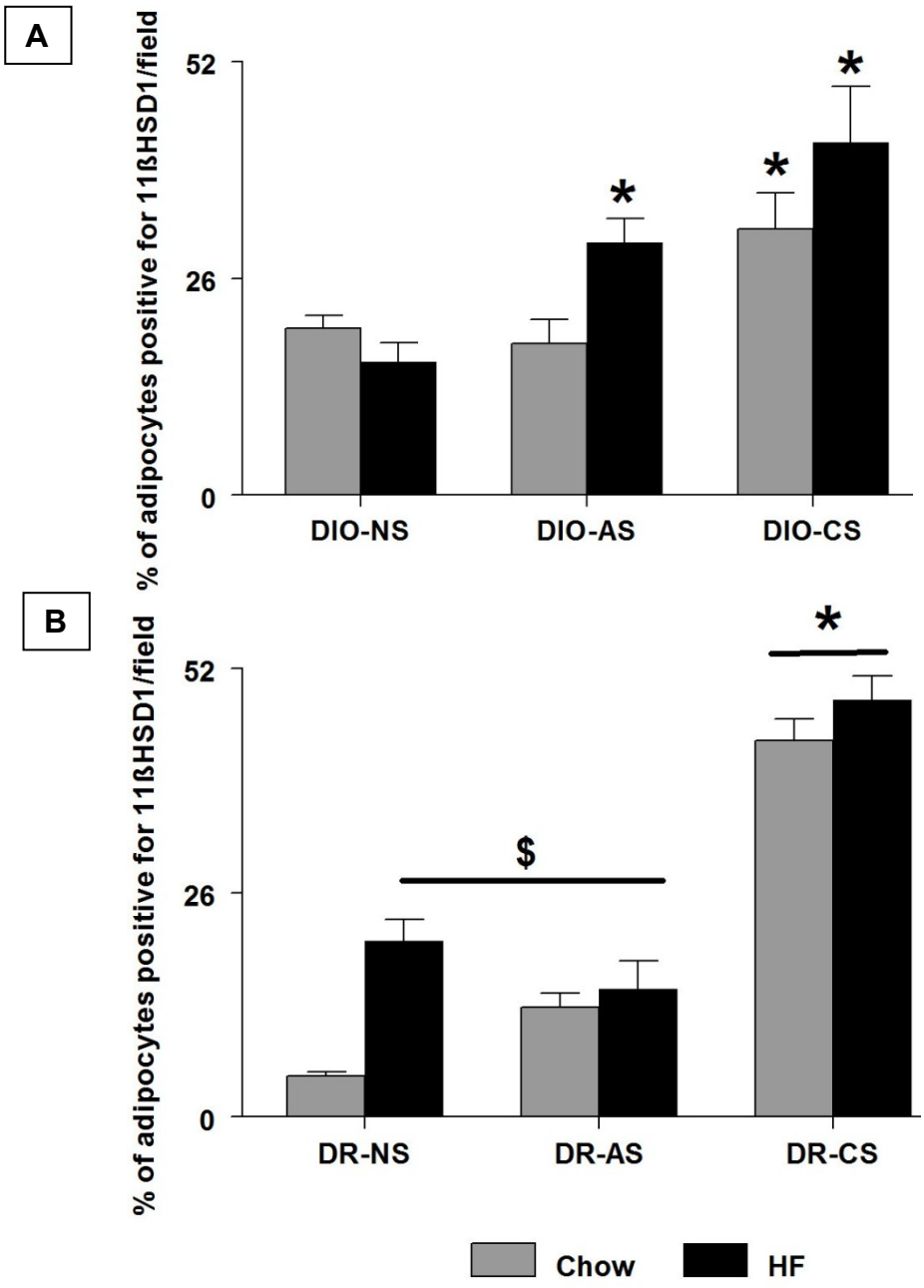

Fig. 6-6 A and B: Effects of prenatal stress and postnatal HF diet exposure on 11βHSD1 protein levels in the VAT

The percentage of adipocytes positive for 11βHSD1 staining per field (20X magnification) in the DIO and DR groups are shown in Fig. 6-6 A and B respectively. '*' denotes significant difference from non-stressed groups, '$' denotes significant difference from DR-NS-chow group.

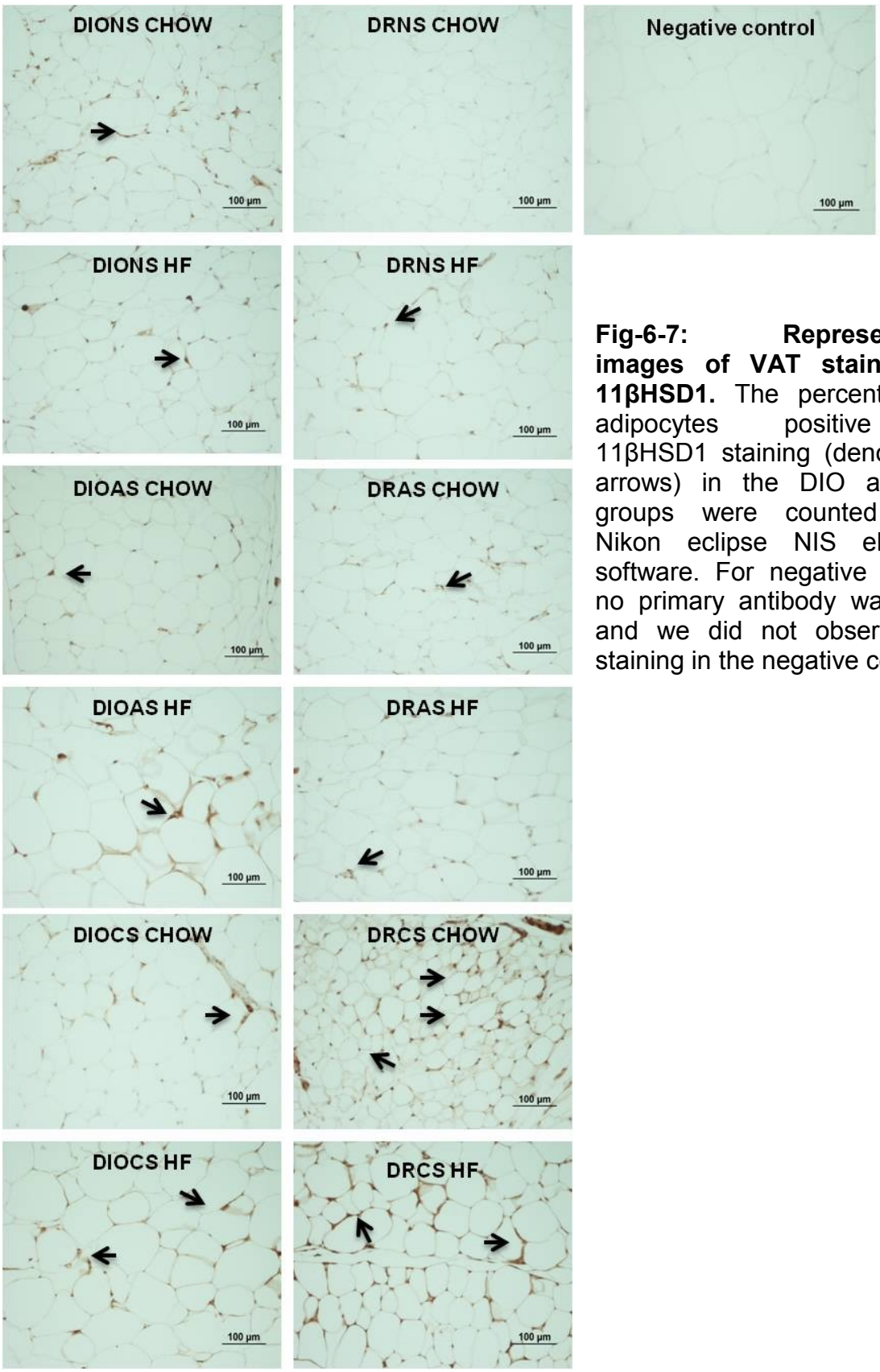

Fig-6-7: Representative images of VAT stained for 11βHSD1. The percentage of adipocytes positive for 11βHSD1 staining (denoted by arrows) in the DIO and DR groups were counted using Nikon eclipse NIS elements software. For negative control, no primary antibody was used and we did not observe any staining in the negative control.

Discussion:

Findings from the previous studies (chapter 4 and 5) suggested that prenatal stress could program the DIO offspring for adverse metabolic outcomes. Further these metabolic changes observed in the prenatally stressed offspring were associated with a hyperactive stress axis as marked by elevated NE levels in the PVN and CRH levels in the median eminence. Despite increased CRH, no changes were observed in circulating CORT levels in the prenatally stressed offspring. Hence, in the present study we wanted to take these findings further and investigate the tissue specific effects of glucocorticoids and examined the expression of 11βHSD1 and GR in the visceral adipose tissue (VAT) and liver. The current findings show that prenatal stress causes tissue specific changes in the expression of 11βHSD1 in both the DIO and DR offspring, a key enzyme implicated in the development of obesity and metabolic syndrome.

Prenatal stress (both acute and chronic) mediated increase in 11βHSD1 mRNA and protein expression was tissue-specific as we observed it specifically in the adipose tissue but not in the liver. Supporting tissue-specific programming of 11βHSD1 expression, studies have shown that dexamethasone treatment during late gestation and gestational diabetes results in offspring with higher 11βHSD1 expression in the liver and adipose tissue [225, 226]. Similarly, postnatal overfeeding, also known to program the offspring for obesity, results in higher 11βHSD1 expression in the adipose tissue [228]. Interestingly, prenatal stress (both acute and chronic) also resulted in increased 11βHSD1 protein expression in the DR offspring, which did not have any adverse metabolic outcomes and stress axis hyperactivation as observed in DIO animals. Whether this has any physiological significance in the DR animals will remain a question

until further experiments on chronic HF diet exposure are carried out. Unlike offspring subjected to acute stress, those that experienced prenatal chronic stress were not obese despite elevated 11βHSD1 expression. These results are supported by previous studies in which maternal perinatal under-nutrition and dexamethasone administration during late gestation also resulted in the offspring with a lean phenotype but with a lipogenic adipose tissue gene profile [225, 229]. Taken together, these studies suggest that elevated 11βHSD1 expression precedes obesity in fetally programmed offspring.

Increase in VAT 11βHSD1 expression in the DIO offspring was accompanied by greater adipocyte size suggesting that adipogenesis occurred through hypertrophy of mature adipocytes and not by hyperplasia of preadipocytes. Glucocorticoids generated intracellularly within the adipocytes through increased 11βHSD1 activity could have mediated hypertrophy of the adipocytes as a recent study has demonstrated reduction in the adipocyte size after treatment with 11βHSD1 inhibitor, carbenoxolone in diet-induced obesity [230]. Several studies have suggested an inverse relationship between adipocyte size in the VAT and insulin sensitivity [231, 232]. Bigger adipocytes tend to shift the balance towards a pro-inflammatory state resulting in increased secretion of pro-inflammatory cytokines and decreased secretion of adiponectin [233]. Greater VAT 11βHSD1 expression, hyperinsulinemia coupled with hypertrophied adipocytes in the prenatally stressed DIO offspring indicate that these animals are probably insulin resistant. Similar to DIO offspring, prenatal stress also significantly increased 11βHSD1 expression in the VAT of DR offspring. However, it did not translate into hyperinsulinemia or hypertrophied adipocytes in the DR offspring suggesting that these

animals have compensatory mechanisms that protect them from these metabolic changes.

Previous studies using glucocorticoids as a programming factor in rodents have demonstrated permanent changes in glucocorticoid receptor expression in various tissues like hippocampus, liver, and adipose tissue [61, 234, 235]. However, in the current study chronic prenatal stress did not affect GR mRNA expression in the adipose tissue, liver and hippocampus (chapter 5). The induction of 11βHSD1 expression in the VAT in the presence of normal circulating cortisol and GR milieu suggests that other factors such as leptin [236], free fatty acids (FFA) [237], insulin [237, 238] or pro-inflammatory cytokines [238] released from the adipose tissue role could contribute to the observed increases in 11βHSD1 expression. It is interesting that we did not observe any additive effect of postnatal HF diet exposure on 11βHSD1 expression in the prenatally stressed offspring as some of the factors known to increase with HF diet like leptin [236] and FFA [237] are known to increase 11βHSD1 expression as well.

There are several mechanisms by which increased 11βHSD1 and the resultant increase in intra-adipocyte CORT could contribute to the development of obesity and metabolic syndrome. In the adipose tissue, glucocorticoids regulate the activity and expression of lipoprotein lipase (LPL) [239], an enzyme expressed in endothelial cells that catalyzes the conversion of circulating triglycerides to FFA, which is then reesterified to be stored in the adipocytes [240]. Higher LPL activity results in more triglyceride accumulation and hypertrophy of the adipocytes leading to metabolically compromised, pro-inflammatory and insulin resistant adipose tissue. This condition is further compounded by the anti-lipolytic actions of insulin during the hyperinsulinemic

state [89]. Glucocorticoids also promote pre-adipocyte differentiation and proliferation resulting in increase in fat mass. In addition to lipogenesis, glucocorticoids can also cause lipolysis by stimulating hormone sensitive lipase in the adipose tissue which increases FFA flux in the portal circulation and thus facilitates ectopic fat distribution in liver and muscle [241]. Further studies need to be conducted in this model to examine these possibilities.

In summary, the present study suggests that prenatal stress could result in upregulation of 11βHSD1 expression in the visceral adipose tissue of the offspring. Although, both DIO and DR offspring had increased 11βHSD1 in the adipose tissue, DR offspring exhibited a healthy metabolic phenotype. Hence, 11βHSD1 could mediate the metabolic effects observed in the prenatally stressed offspring and thus may be a potential mechanism for fetal origins of obesity.

CHAPTER 7

SUMMARY AND CONCLUSIONS

The concept of fetal programming dates back to the early 1950s when it was found that the emotional state of the mother during pregnancy can affect the postnatal behavior of the offspring [242, 243]. However, the concept in its contemporary form with relevance to metabolic and psychological disorders was introduced by Barker and colleagues who reported increased incidence of ischemic heart diseases in individuals from England and Wales whose moms experienced famine during pregnancy at the time of World War II [12]. This observation led Barker to hypothesize that, sub-optimal in utero conditions during fetal development can leave lasting impressions in the offspring and may increase the susceptibility to diseases that may occur decades later. Compromised in utero conditions has been shown to result in low birth weight or intrauterine growth retardation (IUGR) which has been associated with adult diseases like obesity, hypertension and other cardiovascular diseases both in humans and experimental animal models [14-16]. The increased susceptibility to diseases of small sized babies is attributed to altered ontogeny of the organ systems (reduced beta cells, fat cells and nephron) [28, 29], altered neuroendocrine functions [32-35] and epigenetic changes in the genes regulating metabolism [47]. Evolutionarily speaking, these developmental changes could be an adaptive response to cope with limited resources that may be available to the offspring during postnatal life since there is every possibility that the situation that existed during pregnancy could extend beyond that period and there is no way for the fetus to predict otherwise. However, Gluckman and Hanson have an interesting theory that the adaptive responses can become pathological if there is a mismatch between the anticipated and the actual postnatal environment [244]. In Barker's study, the individuals born to undernourished moms were programmed to

survive energy deficient conditions, instead they were exposed to a world with ready availability of high calorie foods. This mismatch probably resulted in increased incidence of coronary artery disease in the investigated population [12]. Hence, prenatal factors do not cause a disease but rather increase the susceptibility to develop diseases when accompanied with an additional postnatal experience like high fat diet exposure. This concept could, or at least in part, explain the increase in the incidence of obesity and type II diabetes in developing countries where poor nutritional status in the prenatal period is combined with the present fast food culture post partum.

Several prenatal factors including over/under-nutrition, stress and exposure to endocrine disruptors have been under investigation in the past decade. For my thesis, I chose to investigate the effects of prenatal stress, as a method to study the role of glucocorticoids in fetal programming of metabolic syndrome in diet-induced obese (DIO) and dietary resistant animal model. To our knowledge, this is the first time DIO/DR rat model is being used in a fetal programming study. Being a polygenetic obese animal model, employing DIO/DR model in our studies will help us understand if there is differential sensitivity to the programming effects of prenatal stress in an obese vs lean maternal population. This is particularly relevant in today's society where 30% of the maternal population is obese [4, 5]. Also, the effects of prenatal stress varied based on the timing, frequency and duration of stress application [176, 177]. Hence, in our studies, we investigated 2 different types of stressors: acute stress (once a week, surgical stress under isoflurane anesthesia) and chronic immobilization stress (restraint stress, thrice a day from day 14-21 of gestation).

In the first study (chapter 4), we characterized the metabolic effects of prenatal stress (acute and chronic) in DIO and DR rats. Chronic but not acute stress resulted in decreased birth weight in the DR offspring when compared to the controls. To begin with, DIO offspring had lower birth weight than DR offspring and stress did not decrease this further. The last stage of gestation is the period of rapid fetal growth which explains why chronic stress lowered birth weight and acute stress did not. Despite no change in birth weight, acute stress significantly increased the postnatal body weight gain and calorie intake. Further, exposure to postnatal HF diet resulted in adiposity and hyperinsulinemia in the acutely stressed DIO offspring. On the other hand, offspring from the chronically stressed dams had catch up growth after weaning to match the body weight of the controls. Low birth weight accompanied with catch up growth in the early postnatal period has been demonstrated to increase the risk for developing obesity and type 2 diabetes in adulthood [245]. Unlike acute stress, HF diet exposure resulted in a lean phenotype in the chronically stress DIO offspring. However, DIO-CS animals on HF diet exhibited hyperinsulinemia, which could lead to the development of type 2 diabetes and cardiovascular diseases. We also observed a significant genotype difference in the programming effects of prenatal stress. Prenatally stressed DR animals did not exhibit adiposity or hyperinsulinemia. If at all, chronic prenatal stress reduced fat mass and leptin levels in the DR animals. These results suggest that there is differential susceptibility to prenatal stress-induced metabolic effects in DIO and DR animals. Another important observation in the present study is that DIO animals, which are polygenetically prone to become obese, have reduced birth weight compared to DR rats which reinstates low birth weight as a predisposing factor for developing obesity

later in life. To my knowledge, this is the first time birth weight has been reported in DIO rats. The body weight of DIO animals surpassed that of DR rats at the time of weaning and it remained greater thereafter, underscoring the importance of the neonatal period in programming energy metabolism and feeding circuits.

The effects of prenatal stress on the phenotype of the offspring are mediated through maternal circulating glucocorticoids as adrenalectomy completely abolishes the programming effects of prenatal stress in the offspring [216]. In an elegant experiment by Barbazanges et al, 2 groups of prenatally stressed offspring were compared in which the corticosterone secretion was blocked by adrenalectomy in one of the groups. The offspring from intact stressed dams had prolonged stress-induced CORT secretion and decreased GR receptors in the hippocampus, whereas the responses were completely blocked in the offspring from adrenalectomized dams [216]. Further, CORT injection in adrenalectomized dams reinstated the effects of prenatal stress in the offspring [216]. The stress protocols used for acute and chronic stress in the present study have been previously shown to increase circulating corticosterone levels in rodents [113, 114]. However, the mechanisms underlying glucocorticoid mediated programming *in utero* is still not clear. One of the most widely held hypothesis is that increased exposure to circulating glucocorticoids leads to IUGR which could happen through multiple ways. Circulating glucocorticoids and activation of sympathetic nervous system as a result of maternal stress can reduce uterine blood flow by increasing the vessel tone [246] thereby reducing the availability of nourishment to the fetus. Also, maternal stress can increase the uterine tone and contractions which on a long-term basis could hamper placental transfer to the fetus [247]. In addition, stress reduces food intake in dams

which also contributes to IUGR. Thus, reduced oxygen and nutrient transfer to the fetus could result in IUGR which has been implicated in the metabolic effects observed in the offspring.

In humans and rodents, there is a protective barrier in the placenta to prevent fetal exposure to excess circulating maternal glucocorticoids. 11βHSD2, an isoform of 11β-hydroxysteroid dehydrogenase enzyme expressed in the placenta inactivates maternal CORT to cortisone and protects the fetus from 2-10 fold higher cortisol levels [62]. While acute stress upregulates 11βHSD2 expression in the placenta, chronic stress did not affect 11βHSD2 activity and thus results in compromising the protective barrier [248]. Further, stress-induced increases in catecholamines have also been shown to downregulate placental 11βHSD2 expression through activation of α-adrenergic receptors [249]. Hence, prenatal stress-mediated fetal exposure to higher levels of glucocorticoids could be the first step in the process of fetal programming of obesity.

There is ample evidence in the literature which suggests an overactive stress axis with impaired feedback regulation in the prenatally stressed offspring with implications for behavioral disorders. Dysregulation of the stress axis has also been associated with the development of metabolic syndrome in the offspring. Hence, in chapter 5 we examined the basal HPA axis activity to know if it is associated with the metabolic alterations observed in the prenatally stressed offspring. Both in DIO and DR rats, chronic prenatal stress increased NE levels in the PVN and CRH levels in the median eminence. On the other hand, acute stress increased NE and CRH only in the DR, but not in the DIOS. The reasons behind the differential effects of acute vs chronic

stress in the DIO animals are not clear. But, previous studies on chronic prenatal stress are in agreement with the present findings where hypothalamic NE levels and limbic CRH levels are higher in prenatally stressed offspring [209] [211]. There are several hypotheses to explain the central activation of stress axis in the prenatally stressed offspring.

1) Impaired negative feedback regulation

Stress axis has a negative feedback mechanism where circulating glucocorticoids bind to its receptors in higher centers of the brain like the hippocampus, amygdala and also act directly in the PVN of the hypothalamus to inhibit further release of CRH and thereby prevent hyperactivation of the stress axis. Previously, studies have reported reduced hippocampal GR and 11βHSD1 expression which contribute to the loss of inhibitory tone leading to hyperactivation of stress axis. However, in our study both acute and chronic prenatal stress did not affect the mRNA and protein levels of GR and 11βHSD1 expression in the hippocampus suggesting that the hippocampus does not play a role in this phenomenon. It is possible that other feedback sites like amygdala might mediate the changes observed in the stress axis.

2) Central leptin resistance

Previous studies from our lab and others have shown that leptin can inhibit stress axis function at the level of the hypothalamus and/or at the level of the brain stem [84, 250, 251]. Leptin binds to its receptors expressed in brain stem noradrenergic neurons and prevents NE release in the hypothalamus. Leptin resistance mediated through decrease in leptin receptor expression [252] or leptin transport across BBB [253] could result in stress axis hyperactivation. In the present study, HF diet exposure resulted in

hyperleptinemia which did not prevent the increase in NE levels in the paraventricular nucleus in the stressed offspring suggesting the possibility of leptin resistance in the stressed offspring. However, further experiments are needed to confirm this phenomenon.

3) Activation of stress axis caused by free fatty acids and cytokines

In addition to leptin, other circulating factors like FFA and pro-inflammatory cytokines like IL-1 and IL-6 can also affect stress axis activity. Both FFA and cytokines have been reported to increase ACTH and cortisol levels [217, 254]. Also, systemic administration of IL-1β increases NE release in the PVN [255]. Hence, it is possible that increase in circulating cytokines or FFA in the prenatally stressed offspring could result in stress axis hyperactivation.

Apart from affecting stress axis activity, NE can directly stimulate feeding behavior. NE infusion or drug-induced release of NE from the brain stem noradrenergic neurons has been shown to increase food intake and facilitate the development of obesity [212, 213, 256]. Interestingly, in the present study we also observed an increase in the CRH levels which is an anorectic peptide. CRH administration has been shown to decrease appetite and also it mediates stress-induced hypophagia [257]. In the present study, acute prenatal stress increased calorie intake in both DIO and DR offspring. However, the opposing effects of NE and CRH make it difficult to directly correlate their effects with the food intake. Food intake regulation is very complex involving an intricate networking of orexegenic peptides (NPY, AgRP, orexin etc) and anorexegenic peptides (CRH, POMC, melanocortin etc) [258]. To make it even more confounding, several neurotransmitters like NE, GABA, 5-HT regulates the neuronal

release of the above mentioned neuropeptides. Hence, apart from NE and CRH there might be other pathways through which prenatal stress could mediate its hyperphagic response.

Despite increases in NE and CRH, there were no differences in circulating CORT levels in the stressed offspring. Although, few studies support our results [169, 215], studies have also reported increase in basal CORT levels in the prenatally stressed offspring [33, 214]. It is illogical to compare the results between the studies due to the differences in stress timing, method of stress application, sex of the offspring and the age of the offspring at which basal CORT levels were measured. Chronic stimulation might result in adrenal gland insensitivity to ACTH and in turn fail to increase CORT secretion. Alternatively, it is possible that the renal clearance of CORT is greater in the prenatally stressed offspring. CORT metabolite measurements in the urine should be carried out to rule out this mechanism. Taken together, the results from chapter 5 suggest that there is dysregulation in the stress axis in both DIO and DR prenatally stressed offspring. However, its relevance to the metabolic changes observed in the programmed offspring needs further investigation.

Not all obese humans have elevated cortisol levels, suggesting alternate pathways behind glucocorticoid-mediated development of obesity [99, 100]. 11βHSD1 is a reductase expressed in the microsomes of metabolically active tissues like liver and adipose tissue and mediates the conversion of inactive 11-dehydro CORT to CORT [92]. Hence, the actions of glucocorticoids are not only dependent on the levels of glucocorticoid receptor (GR), but also on the levels of 11βHSD1 as they regulate the intracellular concentration of CORT. Overexpression of 11βHSD1 in the adipose tissue

mimicked metabolic syndrome with obesity, dyslipidemia, diabetes, hypertension and insulin resistance [102]. On the other hand, inhibition of 11βHSD1 prevented the development of atherosclerosis and diet-induced obesity [103]. These results demonstrate the crucial role for 11βHSD1 in the development of obesity and metabolic syndrome. Hence, we investigated if prenatal stress mediates its metabolic effects by increasing the expression of 11βHSD1 in the liver and adipose tissue.

Both acute and chronic prenatal stress significantly increased 11βHSD1 protein levels in the DIO and DR offspring, but the messenger levels were elevated only in the DIO but not in the DR offspring. The increase in 11βHSD1 is probably not mediated through circulating glucocorticoids as there were no differences in the serum CORT levels and GR expression in the adipose tissue. Alternatively, studies have shown that insulin can affect 11βHSD1 expression both transcriptionally and post-transcriptionally [259]. The fact that protein but not mRNA levels of 11βHSD1 are elevated in the HF-fed chronic stressed offspring suggests that insulin may have post-transcriptional effects in our study. Insulin may increase 11βHSD1 mRNA stability by increasing its half-life [259]. Hence, hyperinsulinemia observed in the acute and chronic stressed offspring might mediate the changes in 11βHSD1 expression in adipose tissue. It is important to note that several other factors like FFA, cytokines and leptin are also involved in modulating 11βHSD1 expression in the adipose tissue.

Increase in 11βHSD1 expression was also accompanied with hypertrophied adipocytes in prenatally stressed offspring (acute and chronic) suggesting that prenatal stress increased triglyceride accumulation in the adipocytes. Exposure to 1 week of HF diet further increased the adipocyte area in the prenatally stressed offspring. Among the

different lipogenic factors, one of the important CORT-induced enzymes is lipoprotein lipase (LPL) [239]. LPL is expressed on the luminal side of the vessels in the adipose tissue. It is involved in the hydrolysis of the circulating triglycerides to FFA which is taken into the adipocytes to be reesterified to triglycerides for storage [240]. Increased LPL in the adipose tissue is associated with obesity and insulin resistance. Besides CORT, insulin also has a stimulatory effect on LPL activity [260]. Since the prenatally stressed offspring have hyperinsulinemia, it is possible that this could have promoted LPL activity as well. Hence, prenatal stress induced lipogenesis is probably mediated through LPL in the adipose tissue.

Alterations in the balance between lipogenesis and lipolysis in the adipose tissue determine the level of fat build up in the adipose tissue [261]. Hence, in addition to lipogenesis, decreased lipolysis can also favor fat accumulation in the adipose tissue. Hormone sensitive lipase (HSL) and adipose triglyceride lipase (ATL) are intracellular lipolytic enzymes that hydrolyze stored triglycerides to FFA which will then be released into the circulation [261]. Decrease in HSL and ATL have been shown to be associated with obesity and insulin resistance. Measuring the levels or activities of these enzymes could prove to be valuable in this model.

Overexpression of 11βHSD1 in the adipose tissue has been demonstrated to increase GC delivery to the liver through the portal vein without affecting the circulating levels of GC's [102, 262]. Increase in 11βHSD1 expression in the adipose tissue in the present study might increase CORT supply to the liver. In the liver, glucocorticoids are involved in gluconeogenesis by inducing the expression of gluconeogenic enzymes like PEPCK and thereby increase serum glucose levels. In our study, we did not observe

any changes in blood glucose levels despite upregulation of GR in the acutely stressed offspring on HF diet. Also, a recent study reinstated the influence of VAT 11βHSD1 in liver metabolism where obesity with increased VAT 11βHSD1 was associated with higher portal CORT levels and non-alcoholic fatty liver disease [262].

Overall, we observed distinct phenotypic differences due to acute vs chronic prenatal stress in the DIO and DR offspring. In the DIO animals, acute prenatal stress resulted in obesity, hyperinsulinemia, increased GR expression in the liver, increased 11βHSD1 expression in the visceral adipose tissue and hypertrophied adipocytes. Chronic prenatal stress also resulted in the same metabolic profile independent of obesity. In addition, they also had partial activation of stress axis. Despite some metabolic alterations like stress axis activation and increased 11βHSD1 expression in the visceral adipose tissue, DR animals appeared metabolically healthy with normal insulin levels and adipocyte profile. However, it is too early to declare that DR animals are resistant to the adverse metabolic effects of prenatal stress as these results were limited to young age group animals and the period of HF diet exposure is acute. Some of the previous fetal programming studies demonstrating obesity and diabetes in the offspring used a chronic postnatal HF diet exposure paradigm [169]. Also, the differences in the effects of acute and chronic prenatal stress might be attributed to the timing and intensity of stress application. Acute stress was applied during early, mid and late gestational period and also milder compared to chronic stress. Chronic stress was more intensive with 3 applications every day during the late gestational period, which is the period of rapid growth. However, the mechanisms underlying the differential responses to acute vs chronic stress is still not clear.

Our results support Barker's hypothesis that the intrauterine environment is a very sensitive period and any perturbations during fetal development can produce long lasting effects in the offspring. Prenatal stress could metabolically program the offspring and increase the susceptibility to develop obesity and metabolic syndrome later in life. Individuals with genetic predisposition to obesity may be more susceptible to the programming effects of prenatal factors. Programming 11βHSD1 expression in the adipose tissue might be a possible mechanism behind the metabolic programming effects of prenatal stress. However, further mechanistic studies are needed to confirm its role in the pathogenesis of prenatal stress-induced metabolic alterations. Other prenatal factors like over-nutrition, under-nutrition etc have also been shown to program 11βHSD1 expression. This suggests that 11βHSD1 might be a common enzyme for many pathways involved in the fetal origins of obesity. The findings in the dissertation recommend new guidelines for pregnancy care in terms of nutrition, workload and also education among the public to create awareness of the long lasting impact of maternal factors on the offspring.

Future Directions

The results from the present study have opened up a lot of new avenues for research to identify the molecular mechanisms underlying prenatal stress-mediated fetal programming of obesity. Transgenerational and paternal inheritance of the prenatal responses in the offspring suggest an important role for epigenetic changes in fetal programming. Hypermethylation changes in the GR and 11βHSD2 gene leading to downregulation of its expression have been previously reported to mediate the effects of prenatal stress in the offspring. Hence, the involvement of epigenetic changes in the

increased expression of 11βHSD1 gene in the adipose tissue needs to be addressed. In the present study, we observed hyperinsulinemia in the prenatally stressed offspring. However, we do not know if these animals develop insulin resistance and type 2 diabetes. Euglycemic clamp experiments are further needed to determine insulin sensitivity in the prenatally stressed offspring. Stress axis and feeding circuits are closely linked as there are neuronal projections between PVN and feed intake regulating centers like arcuate nucleus, lateral hypothalamic area, ventromedial and dorsomedial hypothalamic area. The effects of prenatal stress-induced activation of feeding circuits need to be evaluated. Finally, it is important to address if sexual dimorphism exists in the metabolic phenotype induced by prenatal stress. All the above mentioned studies will provide a complete picture about the mechanisms behind prenatal-stress mediated metabolic programming.

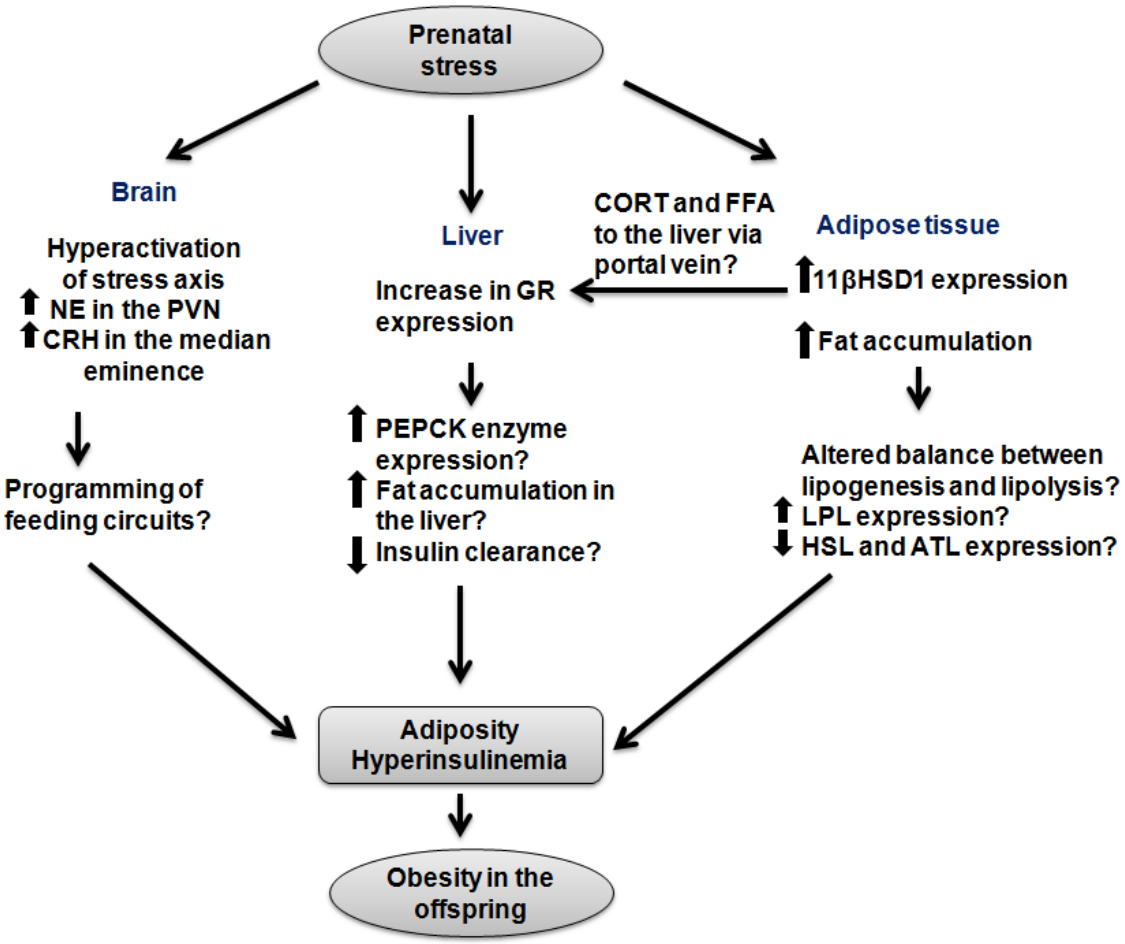

Fig. 7-1: Schematic of conclusions

Prenatal stress results in hyperinsulinemia and adiposity, atleast in part, due to increased stress axis activity and increased 11βHSD1 in the adipose tissue which might predispose the offspring for metabolic syndrome later in life. Genetic predisposition to obesity increases the susceptibility to the metabolic effects of prenatal stress.

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