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DISSERTATION

Effects of maternal dexamethasone treatment early in pregnancy on glucocorticoid receptors in the ovine placenta

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I dedicate this piece of art to my parents and my wife. I am grateful for the unforgettable experiences at the Charité University Berlin, Germany.

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Abbreviations

11β-HSD2	11-β-hydroxysteroid dehydrogenase 2
ACTH	adrenocorticotropic hormone
APS	ammonium persulfate
BET	betamethasone
BNC	binucleate cell
BSA	bovine serum albumin
CAH	congenital adrenal hyperplasia
DAB	diaminobenzidine tetrahydrochloride
DBD	DNA-binding domain
DEX	dexamethasone
dG	days of gestation
ECL	chemiluminescence
hCG	human chorionic gonadotropin
HCL	hydrochloric acid
HPA	hypothalamus pituitary adrenal
Hsp90	heat-shock protein 90
IgG	immunoglobulin G
IUGR	intrauterine growth retardation
GC	glucocorticoids
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRt	total glucocorticoid receptor
GLUT	glucose transportation protein
LBD	ligand-binding domain
L1	level 1
L2	level 2
L3	level 3
MR	mineralocorticoid receptor

NTD	N-terminal transactivation domain
PAG	pregnancy-associated glycoproteins
PBS	phosphate buffer solution
PCNA	proliferating cell nuclear antigen
PGHS-2	prostaglandins H Synthase 2
PL	placental lactogen
oPL	ovine placental lactogen
RDS	respiratory distress syndrome
ROD	relative optical density
SDS	sodium dodecyl sulphate
S.O.	sodium orthovanadate
TEMED	tetramethylethylenediamine

Abstract

In sheep, binucleate cells (BNC) promote fetal growth and are associated with the level of endogenous cortisol. Regulation of the BNC function may be possible via glucocorticoid receptors (GR), and maternal exposure to dexamethasone (DEX) in early pregnancy in early stages of placental development might modify this response. We therefore investigated the expression of GR and its major isoforms as a determinant of these responses. Pregnant ewes carrying singleton fetuses (n=119) were randomized to control (2 ml saline/ewe) or DEX-treated groups (i.m. injections of 0.14 mg/kg ewe weight per 12 h) at 40–41 days of gestation (dG). Placental tissue was collected at 50, 100, 125, 140dG.

Sex-specific differences were found. Only in females, total glucocorticoid receptor protein (GRt) at 50 and 125dG was increased significantly after DEX treatment. In males, GRt protein levels were significantly decreased at 125dG as compared to controls. GRα protein levels were not changed after DEX treatment in either males or females. Three BNC phenotypes were identified with respect to GRα nuclear staining: BNCs with two GRα positive stained nuclei (++), BNCs lacking any GRα staining in the nuclei (- -) and BNCs with one positive and one negative stained GRα nucleus (+-). At 140dG, early DEX treatment significantly increased the proportion of (++) and decreased (--) GRα-BNC as compared to controls. Those effects were sex- and cell type-dependent, indicating the potential of modifying the responsiveness of the placenta to alterations of endogenous cortisol. We propose, according to our findings, that 3 maturational stages of BNCs exist. The overall activity of BNCs is determined by the distribution of these 3 cell types and may become altered through early pregnancy exposure to maternal DEX.

Zusammenfassung

Die Wirkungen von endogenem Kortisol auf die binukleären Zellen (BNC) in der Schafsplazenta, welche für das fetale Wachstum verantwortlich sind, könnten durch Glukokortikoiden Rezeptoren (GR) vermittelt werden und eine antenatale Dexamethason (DEX) Exposition zu Beginn der Plazentaentwicklung, könnte diese Rezeptor vermittelte Wirkung beeinflussen. In der vorliegenden Arbeit haben die Expression von GR als Determinante dieser Wirkungen untersucht. Trächtige Schafe mit Einlingsschwangerschaften (n = 119) wurden entsprechend in Kontrollen (2 ml Kochsalzlösung / kg Körpergewicht des Mutterschafs) und DEX (4 Injektionen von 0,14 mg / kg Körpergewicht des Mutterschafs alle 12 hrs) behandelte Tiere randomisiert. Die antenatale Behandlung wurde an den Tagen 40-41 in der Schafsschwangerschaft durchgeführt. Plazentagewebe wurde bei an den Tagen 50, 100, 125, 140 gesammelt.

Die DEX Behandlung führte lediglich bei den weiblichen Feten an den Tagen 50 und 125, im Vergleich zu den Kontrollplazenten, zu einer signifikanten Zunahme der plazentaren GR-Proteinmenge (GRt). Bei den männlichen Feten zeigte sich hingegen am Tag 125 eine signifikant verringerte GR-Proteinmenge (GRt). Die plazentare GR-Proteinmenge der Isoform GRα war sowohl bei den weiblichen als auch männlichen Feten nach der DEX-Behandlung unverändert. Drei unterschiedliche Phänotypen von BNC konnten anhand spezifischer GRα-Kernfärbemuster identifiziert: BNC mit zwei für GRα positiv gefärbten Zellkernen (++), BNC ohne spezifische GRα Zellkernfärbung (- -) und BNC mit einem für GRα positiv gefärbten Zellkern und einem nicht angefärbten Zellkern (+-).
DEX erhöhte hierbei signifikant am Tag 140 den Anteil (++) und verringerte den Anteil an (- -) BNC im Vergleich zu den Kontrollen. Die Effekte waren geschlechtsspezifisch und BNC-Zelltyp abhängig und beeinträchtigten die Reaktionsfähigkeit der Plazenta auf endogenes Kortisol.
Wir vermuten, dass entsprechend den GRα-Färbemustern 3 Reifungsstadien von BNC existieren und die Gesamtaktivität von BNC durch die Verteilung dieser 3 Zelltypen bestimmt wird, welche möglicherweise durch eine Exposition gegenüber maternaler DEX Therapie in der Frühschwangerschaft verändert wird.

1. Introduction

1.1 General use of glucocorticoids in pregnancy

Glucocorticoids (GC) play a critical role in the treatment of preterm birth for respiratory distress syndrome (RDS). Liggins and Howie first demonstrated that the risk of RDS was significantly reduced by antenatal treatment with corticosteroids before preterm delivery.[1] The mortality and risk of intraventricular haemorrhage in preterm infants were also decreased after antenatal administration of betamethasone (BET) or dexamethasone (DEX) in preterm gestations.[2] These effects extend to a broad range of gestational age and were not race-limited.[3] Babies delivered more than 24 hours (effect starts from eight hours) and less than seven days after initial treatment benefit most.[1]

Other clinical situations which require antenatal GC treatment in pregnancy are maternal asthma therapy with the need for inhalative GC or congenital adrenal hyperplasia (CAH) therapy to avoid possible virilization of the female external genitalia.[4-6] This treatment was reported to prevent or greatly minimize virilization of the external genitalia in CAH girls, but also to have side effects with alteration of brain function and on the programming of the hypothalamus-pituitary-adrenal (HPA) axis.[7]

1.1.1 The pharmacology of glucocorticoids

The endogenous GC (Cortisol) is the primary stress hormone that maintains homeostasis and affects almost all organs and tissues in the body. After activation of the HPA axis, Cortisol is synthesized and released into the circulatory system by the adrenal cortex.[8] GC regulate various biological processes, including cell proliferation, immune system, skeletal development, glucose metabolism, reproduction, cognition and behavior.[9] Synthetic GC are widely used for chronic and acute inflammatory diseases, lymphoid malignancies and organ transplant rejection as a powerful treatment with anti-inflammatory and immunosuppressive actions.[10]

The cellular response to GC varies in magnitude and specificity of action.[11-13] GC decreased the number of lymphocytes which can protect cells of endometrium, liver, mammary epithelium and ovarian follicle.[14] GC sensitivity varies according to the types

of organs and tissues and even stages of cell growth.[15] In addition, tissue-specific GC resistance often develops in patients undergoing long term GC treatment. As side effects (listed in more detail below), GC treatment can also result in osteoporosis, abdominal obesity and growth retardation in children, limiting the therapeutic benefit.[10, 16, 17]

1.1.2 Side effects of glucocorticoids

1.1.2.1 In general

The side effects of GC therapy occur with different prevalence, in different organs, and after different durations of therapy (summarized in table 1). The severity ranges from more cosmetic aspects (e.g. teleangiectasia, hypertrichosis) to serious disabling and even life threatening situations (e.g. gastric hemorrhage).[16] Single or multiple side effects can occur at the same time in one person.[16]

Skin	atrophy, striae rubrae distensae, delayed wound healing steroid acne, perioral dermatitis erythema, teleangiectasia, petechia, hypertrichosis
Skeleton and muscle	muscle atrophy/myopathy osteoporosis bone necrosis
Eye	glaucoma, cataract
Central nervous system	disturbances in mood, behavior, memory, and cognition “steroid psychoses,’’ steroid dependence cerebral atrophy
Electrolytes, metabolism, endocrine system	Cushing’s syndrome, diabetes mellitus, adrenal atrophy, growth retardation hypogonadism, delayed puberty increased Na^+ retention and K^+ excretion
Cardiovascular system	hypertension, dyslipidemia, thrombosis, vasculitis
Immune system	increased risk of infection re-activation of latent viruses
Gastrointestinal	peptic ulcer, gastrointestinal bleeding, pancreatitis

Table 1: GC therapy in adults–overview of possible side effects.

Adapted from Heike Schacke et al.[16]

1.1.2.2 In pregnancy

In repetitive GC dosis regimens, women threatened with preterm delivery are treated with synthetic GC such as BET or DEX to promote fetal lung maturation. Several studies have shown an increased risk of behavioral disorders,[18] elevated blood pressure, and increased insulin resistance[19] together with changes in HPA axis baseline and stress responsiveness in preterm infants exposed to high levels of GC in utero.[20] 30-years follow-up studies of the original Liggins cohort[21-24] show that subjects who had been exposed to BET (24-48 mg total BET dose) as fetuses exhibit more insulin resistance measured by an oral glucose challenge.[22] The incidence of impaired lung function, the prevalence of wheeze and asthma, or the risk of cardiovascular disease were not changed after a single course of BET treatment.[24] In other follow-up studies, renal function at the age of 19 years was retarded after a single course of fetal BET exposure, although this finding may be attributed to preterm birth rather than to early GC exposure, because the incidence of chronic renal failure is increased in prematurely born individuals.[25] Recently, it was reported that GC treatment increased the risk of aortic stiffness as well as changes in glucose metabolism according to a 25-year follow-up study of a cohort of preterm-born individuals from 5 centers in the United Kingdom.[26] These associations suggest that exposure to stress, such as excess exposure to GC and undernutrition, can alter fetal growth and permanently alter body's structure, physiology and metabolism and thereby increase the risk of many kinds of disease in the offspring throughout life leading to the "Fetal Origins Hypothesis".[22] The underlying mechanisms remain to be fully investigated. GR distribution and function may play an important role. Studies have demonstrated that GC have greater effects on brain structures that contain higher levels of GR and mineralocorticoid receptors (MR), such as the limbic system, hypothalamus and cortex. Therefore, GC may program the HPA function by affecting these structures, which are critical for the regulation of HPA function.[27] In intrauterine growth inhibition (IUGR) pregnancies, the promoted placental vascular resistance increased the workload on the fetal heart suggesting that altered placental structure possibly may result in fetal programming of cardiovascular disease.[28] In later life, hypertension and metabolic disease may be programmed by decreased

activity of placental 11-β-hydroxysteroid dehydrogenase 2 (11â-HSD2) activity which increased fetal exposure to maternal cortisol during fetal development.[29] The placenta regulates nutrient transport according to the maternal supply situation. The placenta function itself can be altered by various maternal disturbances such as reduced nutrition, altered cortisol levels and decreased blood flow from uterus to placenta, resulting in changes in the methylation of placental genes and increased placental oxidative/nitrative stress.[30] More and more evidence indicates that the placenta not only responds to disturbances in the maternal compartment but that it also might modulate the programming stimuli to the fetus.[30, 31]

1.2 Role of the placenta

As the conduit between the maternal and fetal circulatory system, the placenta plays a key role in regulating the development of many pregnancy complications. The placenta constitutes an effective interface between the fetal and the maternal environments and regulates fetal growth as a function of maternal physiological alterations.[32] During pregnancy, the placenta maintains fetal homeostasis, provides an immunological barrier between fetus and mother, mediates the transfer of gas, water and nutrients across the placenta, secretes various hormones, cytokines and signaling molecules and regulates utero–placental blood flow.[32] In experimental models, placental morphological structure, transport capacity, and function were more or less affected by maternal disturbances associated with the programming of disease risk, which suggests a link between prenatal adversity and the triggering of fetal adaptive responses.[30] Therefore, it is very likely that the functional ability of the placenta plays an important role for the developing fetus and may determine disease risk and long-term health.

1.3 Sheep as an animal model to investigate the effects of GC on the placenta

Many investigations of human pregnancy cannot be adequately performed for both ethical and practical reasons. The use of animal models has no such limitations and they are in many ways superior to studies in humans: 1. It is easier to select and estimate the

health of mothers before pregnancy; 2. The nutrition intake and environmental conditions are completely under control; 3. Non-invasive techniques (e.g., ultrasound) can be repeated as frequently as necessary, without increasing the burden on mothers (e.g., by eliminating the need to travel to the laboratory); 4. It is easier to perform invasive techniques in animals (e.g., blood sampling and biopsies).[33]

But every animal model has its advantage and disadvantage. As mammals, the mouse and rat are highly related to humans in aspects of genes sequence, signal pathways, organs and physiology.[34] The major concerns are that rodents are altricial animals and born with a depauperate brain and endocrine/paracrine system during the weaning period.[34] Furthermore, in the mouse and rat model, fetuses from the same litter may receive a variable nutrient supply.[34] Primates are the ideal animal model, but they require expensive housing, and their suitability is limited by their lifespan and ethical considerations.

Since it is possible to place catheters in both the maternal and fetal circulatory system, repetitive samples can be taken from non-anesthetized pregnant ewes with continuous investigation of blood factors. Hence, the pregnant sheep has been widely used as an excellent animal model over the past 40 years.[35, 36] Sheep has been proven by several groups as a good animal model to investigate the effects of prenatal GC on tissues and organs.[33, 37-40] Using sheep, the GC was firstly administered to treat preterm delivery by Liggins and Howie and many experiments have been done afterwards using sheep.[1] Secondly, compared to small laboratory animals, the longer gestation period in the sheep is closer to that of humans. As sheep reaches maturity at about 12 months of age, the effects of GC can be easily investigated at different gestational stages.[41]

1.3.1 Sheep placenta

1.3.1.1 Sheep placenta vs. human placenta

Although placental morphology differs among mammalian species, a common structure is the syncytiotrophoblast which is a multinucleate layer of fused epithelial cells forming a functional barrier to transfer nutrients between mother and fetus.[42]

In humans, the placenta consists of a hemochorial discoid type and its cotyledonary villi have direct contact with the maternal blood.[43] In the sheep, 75-120 placentomes, including a placental cotyledonary component and a maternal caruncular, spread over the endometrial layer of the uterus and function as maternal-fetal exchange units.[44]

Although differences between sheep and human placenta have been reported, important similarities in structure and function exist. The structure of the villous tree in both sheep and human placentas are similar and can be recognized as terminal, intermediate and stem villi.[45] Similarly, in both humans and sheep, fetal vessels of cotyledonary villi are comprised of terminal capillaries, intermediate arterioles and venules, and stem arteries and veins.[46] Similarities in function between sheep and humans have been intensively reviewed by Nathanielz et al.[33] It has been reported that placental oxygen and nutrient (glucose and amino acids) consumption, transfer and metabolism rates are nearly the same in both species.[41] Therefore, the sheep placenta is thought to be a reasonable and very important animal model to investigate human placental physiology.

1.3.1.2 Placentomes

In humans, implantation produces a single trophoblast tissue mass forming a single flat disc. In sheep, diffuse implantation produces multiple small masses of trophoblast tissue and forms 75-120 dispersed cotyledons which form the exchange unit “placentome” with maternal caruncle. Caruncles are endometrium areas with glands but they have a vascular organization totally different from that in glandular areas.[47]

Placentome number and size vary greatly among different species, from four to six big ones in deer to 100-150 much smaller ones in cow.[48, 49] While the number of caruncles distributed in the uterus determines the placentome number, the size of the villi determines the placentome size.[48]

1.3.1.3 Placentome classification

Ovine placentomes can be classified into four types by their gross morphological appearance.[50] The fetal face of the placentome is defined by a thin hemophagous zone, where maternal blood bathes between the maternal and fetal villi. This zone appears

black and is inverted inside the round placentome which is defined as A-subtype (Figure 1). Therefore, only a small area of this zone on the external surface of the A-subtype is visible. In the flatter D-subtype placentomes, the hemophagous zone is everted and covers the entire fetal facing surface of the placentome (Figure 1). Between the A and D types there are two more categories, the B- and C- subtypes, which have intermediate degrees of hemophagous zone eversion (Figure 1). Under normal conditions, the A- and B-subtype placentomes predominate throughout gestation and account for more than 60% in total.[51] In late gestation and in multiple pregnancies, the C- and D- subtypes, which are less common than A- and B-subtypes, present with higher frequency.[50] Additionally, the inverted A- and B-subtypes are smaller and lighter than the more everted C- and D- subtypes.[52]

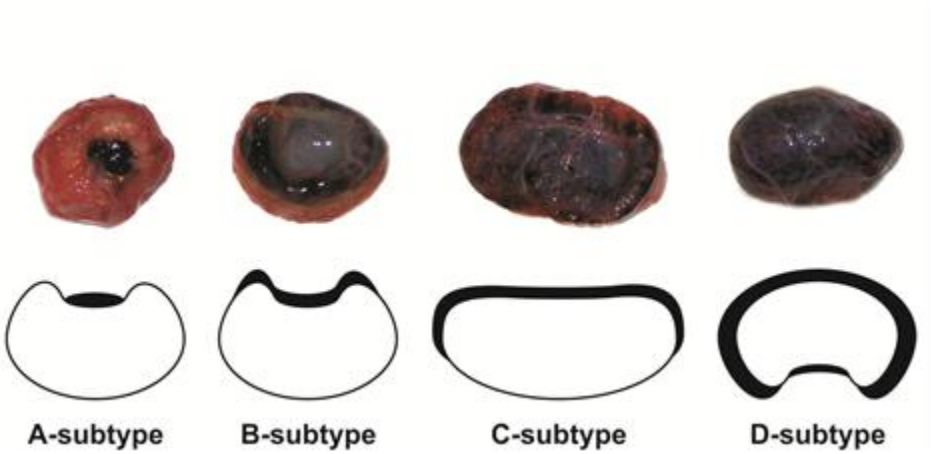


Figure 1: Morphological classification of placentome subtypes.[39]

1.3.1.4 Ontogeny of placentomes

Placentomes are the main site of nutrient transfer, growing rapidly from implantation at 16–30 days to reach a maximum weight at about 75–80 days.[53] Then the weight of placentomes declines near term (145–150 days) and placentomal remodeling takes place, which may help to increase the nutrient-transfer capacity of the placenta during the second half of gestation, when the fetus growth is most rapid.[54] Some previous

studies have reported that the proportion of D-subtype placentomes increased with advancing gestation in control pregnancies.[51, 55] By contrast, Braun et al. found that neither the number of individual subtypes nor the total number of placentomes was altered with gestational length.[39]

1.3.1.5 Functional differences between placentome types

In sheep, the sizes of individual placentomes and the distribution of placentome types are affected by many factors, including alteration in maternal-fetal blood flow, nutrition, O_2-supplement, temperature, and number of sites for implantation.[56] Generally speaking, adverse intrauterine conditions early in gestation lead to a shift from A- and/or B- to C- and/or D-subtype placentomes later in gestation. For instance, after maternal undernutrition or hypoxemia early in gestation, during a phase of rapid placental growth, the frequency of C- and D-subtypes is increased two- to five fold close to term.[51, 56] Therefore it is proposed that the presence of more C- and D-subtype placentomes is a placental regulatory mechanism to increase nutrient supply from mother to the fetus.[57] However, the exact function of these four different placentome types remains controversial and more experiments about placental nutrient delivery with respect to placentome type need to be done. Maternal BET administration in late gestation affected the proportions of placentome subtypes and related expression of prostaglandin H synthase 2 (PGHS-2).[39] However, as indicated above, the data did not support the previous hypotheses that B-, C- and D-subtypes develop from A-subtypes during gestation.[39]

1.3.1.6 Levels within a placentome

The placentome consists of caruncular endometrium and cotyledonary chorioallantois. The medial cross section of a placentomes can be divided into three levels: levels 1-3 (Figure 2). Level 1(L1) includes maternal endometrial crypts, the tips of the cotyledonary villous tree and is located near the endometrium and myometrium interface. Level 2(L2) covers the intermediate area. Relative to myometrium, Level 3(L3) is the most distal area of the placentome including the ends of endometrium and the chorionic plate and is

easily recognized by its dark hematoma (Figure 1+2).

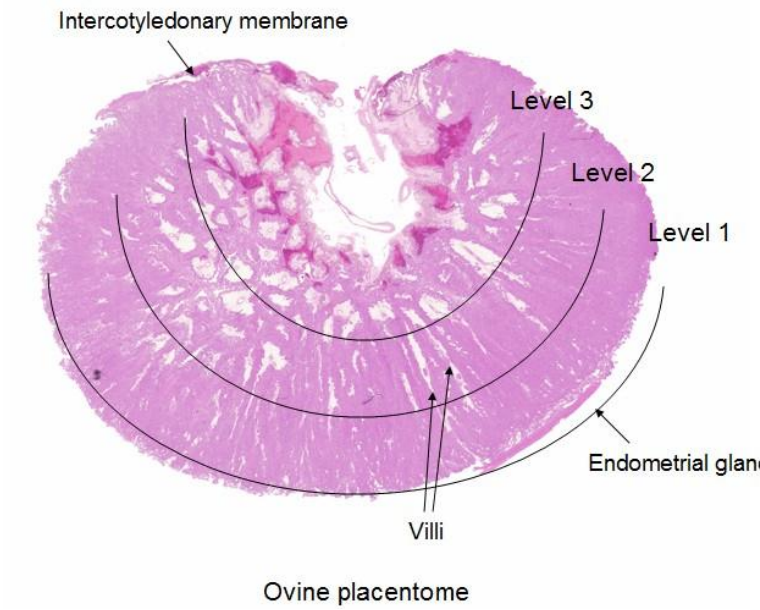


Figure 2: Macroscopic view of a hematoxylin stained placentome.
The placentome is divided into three levels according to the structural differences observed when looking though the microscope to the outside edge. The pale areas are space among villous tissue; in between villi are the maternal sheaths. Adapted from Braun et al.[58]

1.3.1.7 Binucleate cells

In all ruminant placentas, binucleate cells (BNCs) are found in the trophectoderm throughout pregnancy.[59] One theory is that BNCs are formed from uninucleated cells in the fetal trophectoderm, in which the nuclei divide but not the cytoplasm.[60] However, so far, this could not be proven directly. BNCs migrate through the fetal-maternal placental interface to fuse with the maternal epithelium.[61] As a result of the migration, BNCs fuse with a uterine epithelial cell or a syncytial layer, which maintains the feto-maternal hybrid syncytium.[61] At the same time, BNCs deliver the granules by exocytosis from the syncytium into the maternal circulation.[61] The granules contain pregnancy-associated glycoproteins (PAG) and placental lactogen (PL)[62] and therefore play an important role in maintaining the structures and secretions at the feto-maternal interface which may be crucial during pregnancy.[59] oPL is definitely secreted by BNCs into the maternal circulation. The source of fetal oPL is not clear yet. It has been speculated that "…there

may be a constant low level of release for granules prior to BNC migration. Alternatively there may be a constitutive pathway for oPL secretion independent of conventional regulated exocytotic mechanisms…" (Wooding P., Chapter 6: Synepitheliochorial Placentation: Ruminants (Ewe and Cow) in Comparative Placentation, page 161).[47] However, given our own data on BET effects late in gestation and the observed reduction in BNC numbers associated with a decrease in maternal and fetal oPL suggests that the source of fetal oPL are also BNCs.[58]

1.4 The effect of glucocorticoids on the placenta

Many conditions altering gross placental morphology are associated with increased fetal plasma cortisol levels.[63-65] The degree of placental growth inhibition depends on the dose and timing of treatment, the type of GC and the duration of administration.[66]

1.4.1 In humans

Placental width and surface area were significantly decreased after BET administration by -5.5% and -14.7%, respectively, whereas, placental weight was not influenced by BET administration as compared to controls.[64] The number of syncytiotrophoblast nuclei in the placenta was not significantly altered by BET administration either, but the nucleus surface area and syncytiotrophoblast nucleus circumference in central regions of the placenta were significantly increased after BET administration as compared to controls, indicating elevated syncytiotrophoblast activity/function.[64] However, BET administration did not change placental lactogen protein levels or placental lactogen maternal plasma levels at birth.[64]

DEX treatment promoted nuclear maturation, apical microvilli formation, densely distributed organelles, such as secretory droplets and endoplasmic reticulum.[67] DEX treatment promoted syncytiotrophoblast differentiation via elevated human chorionic gonadotropin (hCG) secretion, reduced cytotrophoblast proliferation and elevated expression of 11â-HSD2, which is expressed by the placental syncytiotrophoblast exclusively and which protects the fetus from high maternal cortisol levels.[67]

In human term placental trophoblast cells, placental glucose transportation proteins

(GLUT) and transcripts were down-regulated via reduced placental glucocorticoid receptor (GR) expression, which may contribute to the growth retardation of the fetus.[68] However, both in vitro and in vivo findings suggest that DEX promotes placental system A activity and increases transportation of amino acids from maternal to fetal circulation, which is temporary and indicates a placental adaptation to reduced nutrient transport under DEX exposure.[67]

1.4.2 In animals

In rhesus monkeys, placental weight after GC administration is inhibited to a lesser extent than fetal weight, which suggests placental efficiency was increased despite a moderate reduction in placental weight.[69] Placental glucose transporters were significantly down-regulated in rats and mice.[68] Antenatal DEX administration in rats reduced fetal and placental weight, accompanied with an elevation in apoptosis of trophoblast cells and a reduction in several growth related genes.[70] In rats, placenta growth was retarded after GC administration,[71, 72] which was possibly mediated via inhibition of placental vascularization and reduced expression of placental vascular endothelial growth factor.[72] There was virtually no change in the activity of enzymes related to gluconeogenesis and lipogenesis in the placenta.[73] The limited alterations in placental enzyme activities indicate that placental metabolic stability achieves a protective function toward the fetus.[73]

1.4.3 In sheep

Fetal cortisol levels can be elevated prematurely during late gestation by adverse intrauterine conditions, such as hypoxemia, undernutrition and cord compression.[74] Placental weight was reduced by 25% by chronic maternal cortisol infusion.[52] Moreover, either early or late GC administration to the mother alters the distribution of placentome types closer to term.[52] Experimentally in the sheep, maternal BET administration late in gestation alters the distribution of placentome subtypes by reducing the numbers of the more everted C and D –subtypes.[39, 75] Maternal DEX administration in sheep increased transplacental glucose gradient in pregnancies with a higher number of everted

placentomes,[76] but fetal cortisol administration reduced the placental delivery of glucose and lactate to the fetus.[75] Therefore, it appears that intrauterine conditions that raise fetal GC exposure and regulate placentome subtype distribution may help to maintain placental glucose supply to the fetus in an adverse environment.[77] The number of A- and B-subtypes was most altered by prenatal BET treatment,[39] and the number of C-subtype was increased after DEX treatment, possibly via altered GR protein level.[78]

The decline in numbers of BNCs near term, associated with increased fetal cortisol plasma levels, can be prevented by fetal adrenalectomy.[59, 79, 80] Additionally, the number of BNCs can be reduced by direct fetal cortisol infusion during late gestation.[59] Maternal BET treatment reduced significantly the number of BNCs by 24% to 47% and also changed the normal distribution of BNCs within the placentome compared with controls between 109 and 146 dG at all three levels.[58] Furthermore, the number of BNCs at L3 was significantly lower compared to L1/L2 after BET treatment, which may suggest that new BNCs develop and grow in L1 and BET disrupts the formation of new BNCs L1.[58]

1.5 Sex-specific differences

1.5.1 Clinical background

For a long time, people have noticed that the female fetus has a lower mortality than the male fetus, and after preterm birth, the motor and neurologic functions are more stable in girls than in boys.[81] Perhaps due to sex-specific differences in hormone metabolism during lung maturation, the fetal lung is more matured and functional in preterm female fetuses than male ones.[81] Also, the proportion of cesarean section is higher in women with male fetuses than those with female fetuses, independent of time of gestations and number of deliveries, indicating that female fetuses adapt better to the “stressful event” of labor than male fetuses.[82] Therefore, the underlying mechanism of sex-specific differences in preterm labor rates and may also have important implications for fetal development, fetal adaptation to the intrauterine environment and the process of labor itself.[81]

1.5.2 Experimental data

Many studies have highlighted the sex-specific changes due to adverse maternal environments such as antenatal GC treatment in animal and human studies.[81] To survive adverse environments, it is believed that male fetuses altered their placental function to maintain continued fetal growth which partially contributes to the increased risk of morbidity and mortality in males,[83] whereas female fetuses show an adaptive response, tend to reduce fetal growth to adapt to maternal disturbances.[81] This is consistent with investigations in humans showing that there is a greater incidence of respiratory distress syndrome in male fetuses compared with that in female fetuses.[84]

This hypothesis is also supported by the observations in various animal models. In rats, females have higher basal levels of plasma corticosterone and higher rates of adrenal corticosteroid genesis than males.[85] Additionally, in female rats, plasma corticosterone levels stimulated by adrenocorticotropic hormone (ACTH) or stress were higher.[85] In spiny mouse, placentas from a male fetus showed sex-dependent responses to DEX administration and elevated number of sinusoids associated with changed expression of some regulatory genes as compared to female placentas.[86]

1.5.3 Sheep model

In sheep, we have previously shown sex-specific effects of DEX treatment in early pregnancy, as a model for maternal distress, on fetal and organ weight and function, some of which persisted into later life.[87-89] Furthermore, early DEX administration did not result in growth inhibition in male fetuses, whereas in female fetuses, early DEX administration resulted in a transient growth reduction, which was associated with significantly lower BNC numbers.[90]

The decrease in BNC numbers after synthetic GC treatment or after endogenous cortisol surge near term may result from an imbalance between survival and apoptotic factors, which increased the rate of BNCs apoptosis.[59] GC-induced apoptosis has been reported in the generation of the immune response in the therapy of lymphoid malignancies.[91] Two pathways for apoptosis activation have been found: the extrinsic pathway, dependent on the ligand binding to a “death signal” receptor and the intrinsic pathway,

regulated by the members of the BCL2 family (Figure 3).[92] GC may trigger apoptosis through the extrinsic and/or intrinsic pathway, but both of the two pathways end in the activation of caspase-3, as central initiators and executioners of apoptosis.[93] In the sheep model, early DEX treatment decreased BNC numbers, reduced placental anti-apoptotic factors (proliferating cell nuclear antigen, PCNA) and increased pro-apoptotic factors (Bax, p53), accompanied with a temporarily decrease in fetal growth in females at 40-41dG.[90] However, neither placental oPL protein nor fetal or maternal plasma levels showed corresponding changes at 100dG.[90] This apparent paradox at 100dG may suggest that BNCs increased the output of oPL to maintain placental and plasma concentrations.[90]

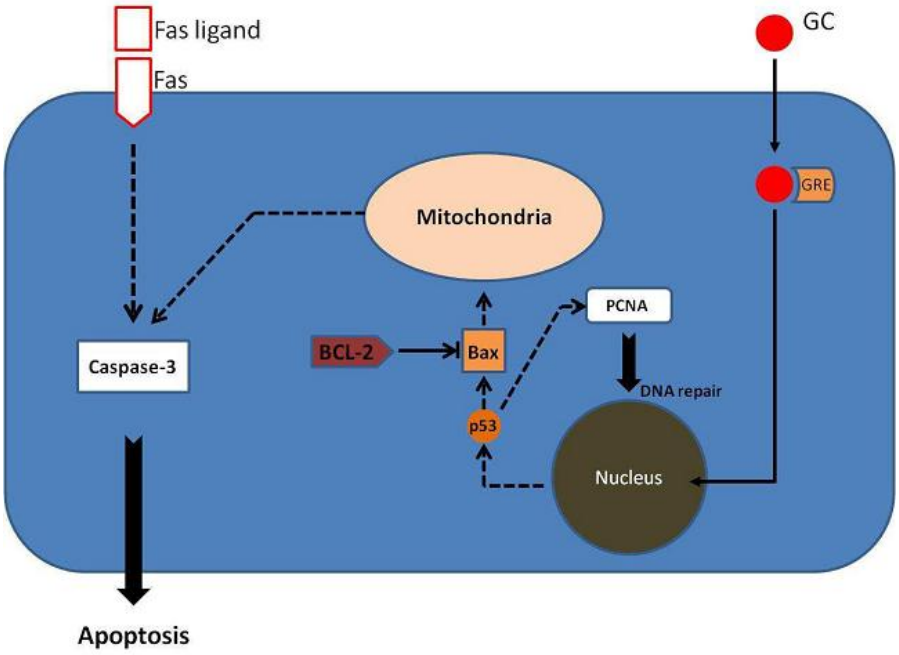


Figure 3: Two pathways for GC-induced apoptosis activation.[90] ENREF 94

Similarly, in sheep, the plasma cortisol levels at 50 dG in control females was already significantly higher than in males, suggesting that females were more responsive to exogenous GC stimulation in early pregnancy than males.[87] Additionally, the adrenal growth of female fetuses in sheep was reduced and the plasma cortisol level of male fetuses was decreased after DEX treatment.[87] In this model of early DEX treatment,

male fetal weight was unchanged by early DEX treatment, while females showed adaptations to DEX treatment.[87]

Although it is still unclear, different adaptive alterations to stress during pregnancy between female and male fetuses may be one explanation for sex-specific adaptation strategies to altered intrauterine environment. In addition, early DEX treatment significantly increased GR mRNA expression levels in females,[87] which also possibly contribute to the sex-specific effect of GC exposure.

1.6 Glucocorticoid receptor

The GR is widely expressed in various organs and tissues and quite important for fetal development and later life.[94] Both the pharmacological and physiological actions of GC are mediated by the GR, therefore, it is necessary to investigate and understand changes of GR.

1.6.1 Glucocorticoid receptors α and β

The GR belongs to the nuclear hormone receptor superfamily which mediates the effects of cortisol within tissues as a ligand-dependent transcription factor.[95] Two isoforms of GR, produced by a single mRNA transcript, have been identified in humans.[96] The α- (777 amino acids) and β-isoform (742 amino acids) share 1–727 amino acids and differ in their carboxy-terminal sequences, as well as in their molecular weights. The ligand-dependent GRα stimulates GC target gene transcription and is accepted as the active receptor isoform.[97] GRβ is ligand-independent and acts as a dominant negative regulator of GRα,[96] although there is some conflicting evidence present in recent literature.[98] Several mechanisms, including competition for transcriptional co-regulators, competition for GC response element (GRE) binding and constitution of inactive GRα/GRβ heterodimers have been suggested to be related with the negative function.[99] Moreover, GRβ may also have an intrinsic gene regulatory ability to alter GC signaling independent of GRα antagonism.[99] Increased levels of GRβ were found in some patients with GC-resistant forms of acute/chronic lymphoblastic leukemia, systemic lupus

erythematosus, asthma, nasal polyposis, and rheumatoid arthritis.[100] The fact that GRβ inhibits the transcriptional activity of GRα suggests that regulation of cellular sensitivity to GC occurs in the expression level of the splice variant.[99] The inactive GR resides in the cytoplasm with heat-shock protein 90 (hsp90) as part of a complex of stabilizing proteins.[101] After binding with ligand, GRα dissolves from stabilizing proteins and forms a receptor-ligand complex followed by a translocation to the nucleus, recognition and binding to the GRE of GC-responsive genes (Figure 4).[102]

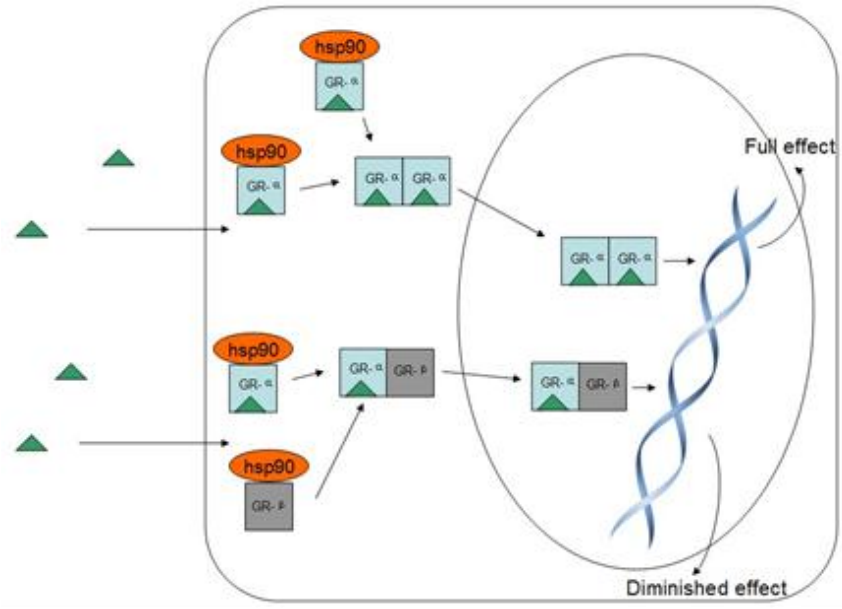


Figure 4: Simplified model of GR-mediated transcriptional modulation.
Redrawn from Bamberger et al.[103]

1.6.2 Other glucocorticoid receptor isoforms

Several additional GR isoforms produced from alternative splicing are found (Figure 5). GRγ was generated from an alternative splice donor site between exons 3 and 4.[104] After binding to GC and DNA, GRγ acts in a manner similar to that of GRα, but it shows a transcriptional model different from GRα on several widely regulated genes.[104-106] GRγ plays an important role in GC resistance in corticotroph adenomas, acute lymphoblastic leukemia in children and small cell lung carcinoma.[106] Other two special GR splice variants, which cannot bind to cortisol, were isolated from GC-resistant multiple

myeloma cells.[107] GR-A was generated from alternative splicing connecting the beginning of exon 8 and the end of exon 4.[99] The function of this isoform is poorly investigated. GR-P, encoded by intron 7, was first classified in a GC-resistant multiple myeloma cell line[107] and has 676 amino acids.[107, 108] GR-P was wildly expressed in various tissues and was the predominant receptor variant in many GC-resistant cancer cells.[109] In several cell types, GR-P has been reported to regulate the transcriptional activity of GRα on GRE according to the cell type.[109-111]

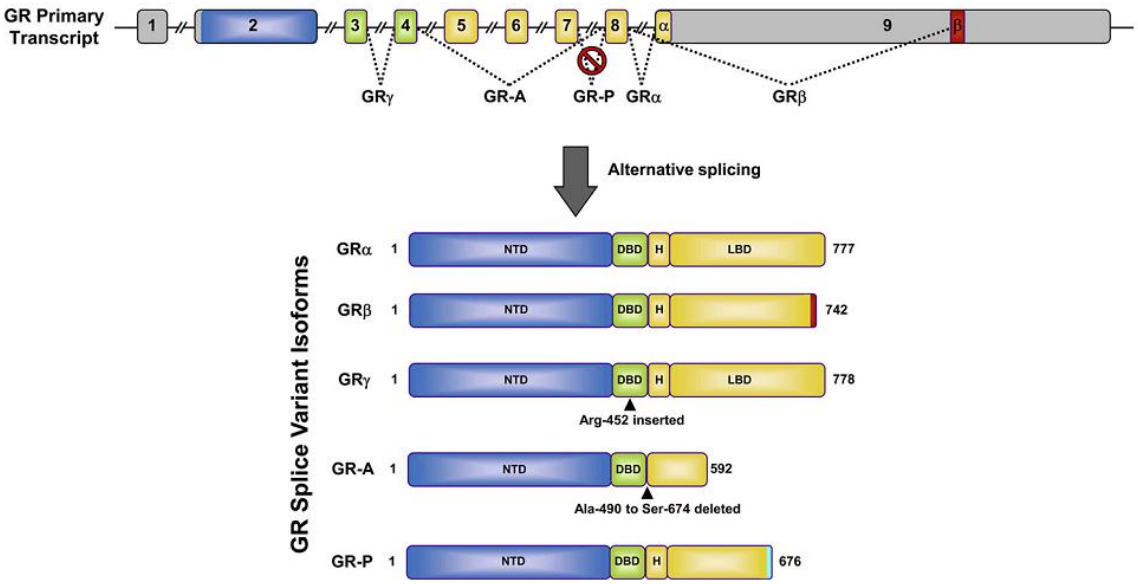


Figure 5: GR isoforms isolated from alternative splicing.
The human GR primary transcript contains 9 exons. The N-terminal transactivation domain (NTD), the DNA-binding domain (DBD) and ligand-binding domain (LBD) were encoded by exon 2, exons 3-4 and exons 5–9, respectively. GRβ is generated from an alternative splicing connecting the end of exon 8 to the beginning of exon 9. GRγ is generated from an alternative splicing between exons 3 and 4. GR-A is generated from alternative splicing connecting exon 4-8. GR-P is generated if the splice exon 7 to exon 8 fails. From Robert H. Oakley.[99]

1.6.3 The effect of glucocorticoids on glucocorticoid receptor

It has been demonstrated that long-term GC treatment down regulates the number of GR in human T cells, Hela cells and JEG-3 cells.[112-114] GC-dependent inhibition of GR may be the reason for the limitation of hormone responsiveness.[115] The cellular mechanisms possibly include down-regulation of GR transcription and the reduction of

GR protein stability,[116] which seems to be cell-type-specific.[115] More than acting as a ligand-dependent transcription factor, GR is under post-translational modification via phosphorylation.[117] As a determinant of down-regulation of GR, phosphorylation has been strongly suggested to alter the receptor responsiveness to GC.[118] Although GC has been demonstrated to down-regulate GR in several tissues, including rat and mouse placentas,[119] the role of GR in human placenta is not well understood.

2. Hypothesis and aim of the study

The placenta, as the conduit between the maternal and fetal environments, may play a central role in regulating the effect of fetal glucocorticoid exposure early in pregnancy during the key phases of placental development.[65, 120] Maternal GC administration in pregnancy has been shown to have long term impact on the health of the affected individual in later life, associated with sex-specific fetal growth inhibition and structural and functional changes in the placenta.[65] Studies in both animals and humans have revealed sex-specific adaptation to a changed environment in utero.[6, 81, 121-127] In contrast to continuous clinical GC treatment of the patient of CAH over several weeks, in the present study, sheep in the first third of pregnancy were given a single course of DEX treatment of 4 x 0.14mg/kg maternal body weight at 12hrs intervals. Maternal DEX therapy did not result in growth inhibition in male fetuses, whereas in female fetuses, DEX administration was associated with a transient growth reduction with significantly lower BNC numbers and increased apoptotic markers, which was not reflected in changes in placental oPL protein levels.[87, 128] The underlying mechanisms remain unknown and the purpose of the current study was to reveal the intermediate role of GR in regulating BNC function and placental development after DEX treatment in early pregnancy. Therefore, we investigated the effects of endogenous cortisol levels and exogenous early maternal DEX administration on the localization and distribution of ovine placental GR and thus evaluated the functional role of GR in the placenta for fetal programming. Based on our previous studies, we hypothesized that the effects of early maternal DEX administration on GR would be sex-specific and placentome subtype-dependent.[39, 128-130]

3. Materials and Methods

3.1 Materials

3.1.1 Chemical substances

Chemical substances/ Article number	Manufacturer
Acrylamide/Bisacrylamide 30% A3574	Sigma-Aldrich, USA
Ammonium persulfate (APS) 09913	Sigma-Aldrich, USA
Avidin/Biotin complex (ABC Kit) PK-4001	VECTOR Laboratories, USA
Bovine serum albumin (BSA) A9418	Sigma-Aldrich, USA
Citric acid monohydrate 00244	Merck, Germany
Coomassie brilliant blue B0770	Sigma-Aldrich, USA
Dexamethasone H02AB02	Mayne Pharma, Australia
Deoxycholatic acid D6750	Sigma-Aldrich, USA
Diaminobenzidine-Tetrahydrochloride (DAB) 189-0	Aldrich, Germany
Distilled water, Mini-Plasco	B.Braun, Germany
Distilled water, Ampuwa	Fresenius Kabi, Germany
Donkey anti-rabbit, IgG (H+L), polyclonal, HRP, 31458	Thermon scientific, USA
Donkey anti-rabbit, IgG (H+L),polyclonal, 711-066-152	Jackson ImmunoResearch Europe Ltd.,UK
Ethanol 200-578-6	Herbeta Arzneimittel, Germany
Fast red K5005	Dako Real Detection System, Denmark
Fast red ab64254	Abcam, UK
Glycine 50052	Sigma-Aldrich, Germany
Goat anti-rabbit, polyclonal, PK-4001	Vector laboratories, USA
GRα blocking peptide sc-1002 P	Thermon scientific, USA
GRβ blocking peptide PEP-222	Thermon scientific, USA
Hydrochloric acid (HCL) 109058	Merck, Germany
Laemmli sample buffer 161-0737	Bio-Rad, Germany
Mayer's hematoxylin solution 089K4342	Merck, Germany
β-Mercaptoethanol M3148	Merck, Germany
Methanol 4627	Sigma-Aldrich, Germany
Microscopy entellan 07961	Merck, Germany
Mouse anti-GRt, monoclonal, MA1-510	Thermo scientific, USA
Mouse anti-β-actin, monoclonal, monoclonal 107K4800	Sigma-Aldrich, USA
Normal goat serum S-1000	VECTOR Laboratories, USA
Pentobarbitone	Valabarb, NSW
Peroxidase (H_2O_2) 8070	Roth, Germany
Pierce enhanced chemiluminescence (ECL) western blotting substrate, 32209	Thermo scientific, USA
Ponceau S dye 141194	Sigma-Aldrich, USA
Protease inhibitor cocktail tablets, EDTA-free 11836170001	Roche Applied Science, USA
Protein assay 500-0006	Bio-Rad, Germany
Protein ladder, BenchMark™, 10748-010	Life Technologies GmbH, Germany
Protein ladder, Page Ruler, sm0671	Fermantas, Thermo scientific, USA
Rabbit anti-GRα, polyclonal, GR(P-20):sc1002	Santa Cruz, USA
Rabbit anti-GRβ, polyclonal, PA3-514	Thermo scientific, USA
Rabbit anti-mouse, polyclonal, 127K4847	Sigma-Aldrich, USA
Rabbit anti-oPL, monoclonal	Provided by Sloboda et.al.
Rabbit anti-caspase 3, polyclonal, AF835	R&D Systems, Australia
20% Sodium dodecyl sulphate (SDS), aqueous solution(w/v) 54661	Biomol, Germany
Skimmed milk powder 70166	Roth, Germany
Sodium chloride 106404	Merck, Germany

Sodium orthovanadate (S.O.) S6508	Sigma-Aldrich, USA
di-Sodium hydrogen phosphate dihydrate (Na_2HPO_4 x $2H_2O$) 106580	Merck, Germany
Sodium dihydrogen phosphate monohydrate (NaH_2 PO_4 x H_2O) 106346	Merck, Germany
Streptavidin–alkaline phosphatase, S2890	Sigma-Aldrich, USA
Tetramethylethylenediamine (TEMED, $C_6H_{16}N_2$) 2067.2	ROTH, Germany
Tri-sodium citrate dihydrate ($C_6H_5Na_3O_7$ x $2H_2O$) 106448	Merck, Germany
Running-buffer (10x) 161-0772	Bio-Rad, Germany
Tris A4112	ROTH, Germany
Tris-HCL (pH 7.5) 214-684-5	Roth, Germany
Tween® 20 P1379	Sigma-Aldrich, USA
Xylene 18118	J.T.Baker, USA

Table 2: General chemicals

3.1.2 Solutions for immunohistochemistry

Solutions	Components
ABC	1000 µl PBS (pH 7.5) + 5 µl Citrate buffer
Citrate buffer	38ml Solution A+162ml Solution B
DAB	25 mg DAB + 10 ml Tris-HCl buffer
2% Normal goat serum	1:50 diluted with PBS (pH 7.5)
Peroxidase-Blocker	65 ml PBS (pH 7.5) + 7.5 ml Methanol + 2 ml H_2O_2
Phosphate buffer solution (PBS, pH 7.5, 2L)	0.4g $NaH_2PO4.H2O$+2.75g $Na_2HPO4*2H_2O$+17.85g NaCl+ distilled water
Primary antibody: Mouse anti-GRt	1:50 diluted in 2% normal goat serum
Primary antibody: Rabbit anti-GRα	1:100 diluted in 2% normal goat serum
Primary antibody: Rabbit anti-GRβ	1:10 diluted in 2% nomal goat serum
Primary antibody: Rabbit anti-oPL	1:20000 diluted in 2% nomal goat serum
Primary antibody: Rabbit anti-caspase-3	1:1000 diluted in 2% nomal goat serum
Secondary antibody: Donkey anti-rabbit	1:200 diluted in 2% nomal goat serum
Secondary antibody: Goat anti-rabbit	1:200 diluted in 2% nomal goat serum
Solution A	21.01g Citric acid monohydrate in 1000 ml distilled water
Solution B	29.41g Sodium citrate dihydrate in 1000 ml distilled water
Tris-HCL (pH 7.0)	121.14 g Tris in 1000ml distilled water titrated with HCL to pH 7.0

Table 3: Solutions for immunohistochemistry

3.1.3 Solutions for western blot

Solutions	Components
Ammonium persulfate (APS) 10%	0.1 g Ammonium persulfate + 1 ml distilled water
Blocking solution 5%	5g milk powder + 100ml PBS-T (pH 7.5)
Blocking solution 7.5%	7.5g milk powder + 100ml PBS-T (pH 7.5)
BSA solution	1 mg BSA + 1ml distilled water
Loading gel 4%	Distilled water6.1ml+0.5M Tris (pH 6.8) 2.5ml+10% SDS 0.1ml+30% Bis-Acryl 1.3ml+10% AP 50 µl+TEMD10 µl
β-Mercaptoethanol in laemmli sample buffer	1ml β-Mercaptoethanol+19ml Laemmli sample buffer
PBS-Tween (pH 7.5)	0.4g $NaH_2PO_4xH_2O$+2.75g Na_2HPO_4 +17.85g NaCl + 2ml TWEEN + 1000ml distilled water
Protein assay	1:5 diluted with distilled water
Primary antibody: Mouse anti-GRt, MA1-510, Thermo scientific, USA	1:100 diluted in 5% normal goat serum
Primary antibody: Rabbit anti-GRα, GR(P-20):sc1002 , Santa Cruz, USA	1:100 diluted in 5% normal goat serum
Primary antibody: Rabbit anti-GRβ, PA3-514, Thermo scientific, USA	1:100 diluted in 5% normal goat serum
Primary antibody: Mouse anti-β-actin, 107K4800, Sigma-Aldrich, USA	1:20000 diluted in 5% normal goat serum
RIPA-Lysis buffer (pH 8.0)	1 ml Triton-X-100 + 0.1g SDS + 0.877g NaCl + 0.242 g Tris + 0.5 g Deoxycholatic Acid + 100 ml distilled water (Before using: 10ml RIPA-Lysis buffer + 5µl S. O.+1 Tablet Proteinase inhibitor)
Secondary antibody: Donkey anti-rabbit, 31458, Thermo Scientific, USA	1:1000 diluted in 5% normal goat serum
Secondary antibody: Rabbit anti-mouse, 127K4847, Sigma-Aldrich, USA	1:10000 diluted in 5% normal goat serum
Separating gel 8%	Distilled water8.9ml+1.5M Tris (pH 8.8) 5.0ml+10% SDS 0.2ml+30% Bis-Acryl 5.3ml+10% AP 100µLTEMD 10µL
Transfer-buffer (pH 8.1-8.4) 2L	6.06g Tris +28.8g Glycine +400mL Methanol+ distilled water
Tris 0.5 M (pH 6.8)	6.057g Tris + 100ml distilled water
Tris 1.5 M (pH 8.8)	18.17g Tris + 100ml distilled water

Table 4: Solutions for Western blot

3.1.4 Equipment

Equipment/Type	Manufacture
Camera, Color video camera 3CCD	Sony, Japan
Centrifuges, 5840R	Eppendorf, Germany;
Centrifuges, Biofuge 13	Heidolph, Germany
Distilled water producer, Elix clinical	Millipore, USA
Electrophoresis-equipment, Mini Protean Tetra system	Bio-Rad, Germany
Electrophoresis-power, PowerPac	Bio-Rad, Germany
Embedding center, Shandon Histocentre 2	ThermoFisher Scientific, USA
Freezer, -85C, U57085	New Brunswick Scientific, USA
Imaging densitometer, VersaDoc Imaging system 4000	Bio-Rad, Germany
Knife, Surgical disposable scalpels	B.Braun, Germany
Magnetic stirrer, MR2002	Heidolph, Germany
Microplate reader, EL800	BioTek Instruments GmbH, Germany
Microscopes, Axioskop	Zeiss, Germany
Microscopes, CTR MIC	Leica, Germany
pH-meter, 766 Calimatic	Knick Elektronische Messgeräte GmbH, Germany
Rotation microtome, HM340E	Microm International GmbH, Germany
Shaker, Polymax 1040	Heidolph, Germany
Shaker, REAX 1 DR	Heidolph, Germany
Shaker, Schüttler MTS	IKA, Germany
Sterilization	Webeco, Germany
TissueLyser, II-85300	Qiagen, Germany

Table 5: Technical equipment

3.1.5 Software applications

Software/Version	Manufacture
Image J 1.48u	National Institutes of Health, USA
Quantity one 1-D software 4.5.2	BioRad, USA
Image system, SPOT	Vistron system GmbH, Germany
Spss 20	IBM, USA
geNorm visual basic application 3.5	Biogazelle NV, Belgium

Table 6: Software applications

3.2 Methods

3.2.1 Animal model

The procedures and animal experiments described below were performed by Dr. Thorsten Braun and colleagues in Western Australia between 2004 and 2007 (Animal Welfare Act 2002 Licence U12/2005, Animal Ethics Committee AEC No. 6-04-40) and frozen tissue were sent to Berlin for further analysis.

3.2.2 Procedures

Pregnant merino ewes (Ovis aries) with singleton pregnancies (total n=119) of known gestational age were randomly divided into control and DEX groups. A total of four maternal intramuscular DEX injections were given to ewes in the DEX group in a dose of 0.14 mg/kg ewe weight, with one intramuscular injection being given every 12 hours over 48 hours on 40–41 dG (term150dG, Figure 6). Control animals received saline injections of a comparable volume (2 ml saline/ewe).

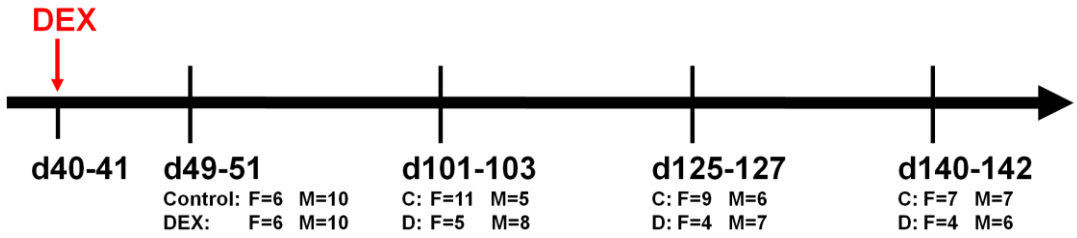


Figure 6: The protocol of DEX treatment and sampling.[87]

On the day of sacrifice, the ewes' weights were measured and blood was collected by venipuncture. After fetuses were sacrificed, fetal body weight and dimensions were measured immediately and tissues were collected after all organs were weighed. Fetal blood gas, metabolite and electrolyte content were measured in samples of umbilical arterial blood. Maternal and fetal blood was centrifuged for biochemical analyses of plasma.

3.2.3 Delivery of lambs and postnatal experimentation

Ewes allowed to deliver were transported to the Medina RSU at about 70 days of pregnancy; they were held in small paddocks for delivery, where close monitoring of ewes and lambs was possible. Management of lambs was performed using usual husbandry practices (e.g. weaning, castration of males, tail docking, immunisation). Every possible effort was made to avoid situations that would be stressful for the ewes (including flock movement to the RSU).

3.2.4 Placenta collection

Experimental treatment to the pregnant ewes was given at the Mt Barker RSU. Ewes were killed by captive bolt and the fetus delivered by Caesarean section. The umbilical cord was clamped and fetuses were killed immediately by injecting an overdose of pentobarbitone into the umbilical vein. Blood samples were collected from the placental cord. Lambs were weighed and measured and their organs were dissected and collected for subsequent analysis.

3.2.5 Immunohistochemical examinations and cell counting

3.2.5.1 Localization of GRt, GRα, GRβ

5µm sections were cut from the middle of whole and fixed placenta embedded in paraffin using rotation microtome.[58] The sectioned tissue was mounted on glass slides and incubated at 37°C overnight to make sure the sections stick to the slide. Slides were deparaffinized with xylene two times for five minutes and rehydrated in a graded ethanol series (100%,90%,70% and 50%) for two minutes each followed by five minutes washing in distilled water and 10 minutes washing in PBS. For antigen retrieval, the sections were incubated in boiled citrate buffer for 20 minutes in a pressure-cooker followed by three two-minute washes in PBS. To block endogenous peroxidase, the sections were incubated with peroxidase-blocker for 10 minutes, followed by a 3*5 minutes washing in PBS. To block non-specific binding, the sections were incubated for 30 minutes in a blocking solution containing 2% normal goat serum diluted in PBS. Afterwards, primary antibody (anti-GRt,[78] anti-GRα[131] and anti-GRβ [131,132]), diluted in 2% normal goat serum,

was dripped onto each section of tissue and incubated at 4°C overnight. According to the antibody information sheets provided by the company, the GRα and GRβ antibodies do not cross-react with the GRβ and GRα antigens. For negative control, the sections were incubated in PBS excluding the primary antibody. After overnight incubation, the sections were washed in PBS for 3*5 minutes and incubated for one hour with secondary anti-rabbit antibody. The sections were washed in PBS for 2*5 minutes before incubating slides with avidin-biotin solution (1µl avidin+1µl biotin+250µl PBS) from Vectastain ABC kit for 30 minutes, followed by 2*5 minutes washes in PBS. For the chromogen, slides were incubated with 3,3-diaminobenzidine tetrahydrochloride (DAB) diluted with Tris/HCL and H_2O_2 for 10 minutes. The sections were washed in Tris/HCL buffer for 10 minutes, in PBS for five minutes and in distilled water for five minutes. Afterwards, the sections were counterstained in hematoxyllin for 60 seconds, followed by a 15 minutes washing step under tap water to remove excess stain. The sections were then dehydrated in a graded ethanol series (50%, 70%, 90% and 100%) two minutes for each step and washed in xylene for 10 minutes. Slides were covered using cover slips with mounting medium.

3.2.5.2 Localization of caspase-3

Procedures were the same as 3.2.2.1 before the sections were incubated with polyclonal rabbit anti-human active caspase-3 diluted in 2% normal goat serum at 1:1000.[133] The sections were then incubated with biotinylated anti-rabbit immunoglobulin G (IgG) antibody at 1:200. Afterwards, the sections were labeled with streptavidin–alkaline phosphatase and developed with Fast red.

3.2.5.3 Co-localization of GRα and oPL

First, the sections were incubated with primary antibody (anti-GRα) and secondary antibody, followed by chromogen step using DAB. Then the sections were washed in Tris-HCL (pH=7.5) for five minutes and in PBS for 3*5 minutes before being incubated with primary antibody (anti-oPL) and secondary antibody. Fast red was then used as chromogen.

3.2.5.4 Co-localization of GRα and caspase-3

First, the sections were incubated with primary antibody (anti-GRα) and secondary antibody, followed by chromogen step using DAB. Then the sections were washed in Tris-HCL (pH=7.5) for five minutes and in PBS-T for 3*5 minutes, followed by incubation with primary antibody (anti-caspase-3) and secondary antibody. Fast red was then used as chromogen.

3.2.5.5 Cell counting

Prepared slides were examined under a microscope at 20*magnification, and digital images of the sections were taken with a camera and the matched software when placental tissue covered more than 80% of the selected field of view. [58] Two sections from each animal were analyzed. To decrease the possibility of false positive counts, GRα BNCs were counted only if more than 30% of the BNC cytoplasm was visible.[58]

A total of 18 random fields of view per section, arranged in three levels were counted after immunostaining (Figure 7). Two sections were manually counted for each placentome and over 6500 pictures in total were analyzed.

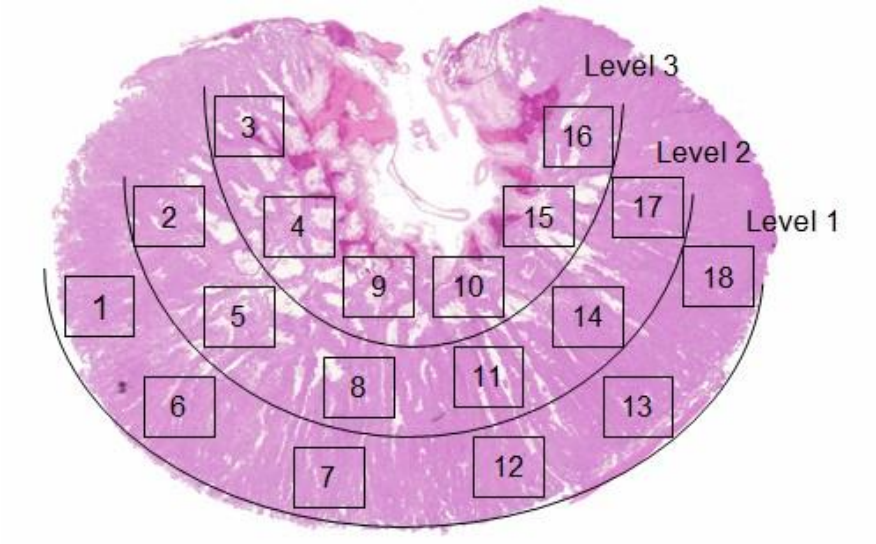


Figure 7: Macroscopic view of a hematoxylin stained placentome.
A total of 18 random fields of view in three levels per section were counted. Adapted from Braun etc.[58]

3.2.6 Protein measurements

3.2.6.1 Protein extraction

Extraction of placenta proteins was done by RIPA-lysis buffer with freshly added protease inhibitors using an oscillator. About 100mg frozen tissue was cut down using surgical knife on ice and then was transferred to a fresh centrifugal tube with 400µl RIPA-lysis buffer containing protease inhibitors. One steel ball was put in every centrifugal tube and then inserted into two special racks. The cover of each centrifugal tube was closed tightly. Then racks were mounted on TissueLayser II (Quiagen). Program was set to 30 hertz for 3*5 minutes (after each time, two racks' sides were changed). The supernatant (containing tissue protein) was then transferred into fresh centrifugal tubes by pipetting.

3.2.6.2 Determination of protein concentration

Serial dilutions of a bovine serum albumin were prepared in distilled water to establish a standard curve. 10µl of the series of standards mixed with 200µl Bradford solution (for background subtraction) was loaded onto a 96-well plate in duplicates. Solutions were measured at 595nm in a microplate reader and the standard curve was generated automatically.

In order to make sure that the same quantity of sample was loaded onto the gel, the protein concentration was measured according to the Bradford method. For this method, 1µl of each sample was transferred into 99µl of Bradford solution. The protein binding to the Coomassie Brilliant Blue changes the dye maximal absorption from 465 to 595nm which linearly correlates with the protein concentration. Therefore, the protein content was determined by measuring absorption at 595nm and calculating the concentration from the standard curve obtained with known BSA dilutions.

3.2.6.3 Sample arrangement

To avoid/minimise the error between different experiments, samples with all factors (dG, sex, placenta type, groups) were selected in one gel with 15 wells as many as possible.

Additionally, one sample from the control group with clear and sharp bands in preliminary experiment was used as an internal control in every gel.

3.2.6.4 Sodium dodecyl sulphate polyacrylamide gel electrophoresis, SDS-PAGE

First, the components of the separating gel were prepared and mixed in a clean glass measuring cup. The gel was poured between two glass plates assembled on the casting stand. Several drops of distilled water was slowly pipetted onto the surface of separating gel to isolate from air (making sure the surface between distilled water and separating gel is horizontal) and the gel was left to completely polymerize for 45 minutes at room temperature. Afterwards, the water layer was poured out and completely removed with the edge of paper. Afterwards the stacking gel was made and mixed and carefully poured onto the separating gel. Then a clean plastic comb with 15 lanes was inserted slowly into the stacking gel, avoiding the formation of air bubbles. After 30 minutes' polymerization, the comb was removed from the stacking gel. The gel and glass plates were assembled and put into the electrophoresis chamber filled with running buffer. It was ascertained that the direction of the electrode was right. According to results of the preliminary experiments, 80µg of protein was mixed with the same volume of sample buffer and the mixtures were incubated at 55°C for 10 minutes and then pipetted into the lanes of the stacking gel (from the 2^{nd} to the 14^{th} lanes). 3µl of the protein marker was loaded into the first lane of each gel to quantify molecular weights of proteins. Electrophoresis was performed at 60 volt for 20 minutes to gather and concentrate the proteins on the junction of stacking gel and separating gel. And then proteins were run at 160 volt for about one hour for separating. When the dye reached the bottom of the separating gel, electrophoresis was stopped and the gel units were disassembled for Western blotting.

3.2.6.5 Western blotting

Transfer

In order to detect the proteins with antibodies, they were moved from within the gel onto a membrane made of nitrocellulose using the method of Western blotting. A transfer

stack with filter papers, sponges, membrane and gel were arranged according to the manufacture's manual. The entire stack was placed in blotting chamber full filled with transfer buffer and one ice stack. The chamber was put into a small box and surrounded with ice. Then the proteins were transferred from the gel onto the nitrocellulose membrane using electric current at 110 volt for 70 minutes, during which the proteins maintained the organization they had within the gel. The effectiveness of transfer was checked by staining the membrane with Ponceau S dye for two to five minutes upon gentle shaking at room temperature. Ponceau S dye was then washed off by washing the membrane several times in distilled water until the dye was completely washed off.

Blocking non-specific protein

The blocking of non-specific binding is performed by placing the membrane in a blocking solution consisting of 7.5% Skimmed milk powder and PBS-Tween overnight at 4°C with constant gentle agitation. The blocking solution reduced the background by binding non-specific proteins to the antibodies.

Incubation with antibodies and detection

The membrane was incubated in either a clean plastic box with 10ml dilute solution of primary antibody or in sealed bags with 5ml dilute solution (5% skimmed milk powder diluted in PBS-Tween) and primary antibody (the purpose is to save antibody which needs high concentration) overnight at 4°C with gentle agitation. The primary antibody solution was discarded and the membrane was washed five minutes in PBS-Tween for five times, followed by incubation in a clean plastic box with secondary antibody in 10ml dilute solution for one hour at room temperature with gentle agitation. The secondary antibody solution was discarded and the membrane was washed five times for five minutes in PBS-Tween.

For the chemiluminescence reaction, the membrane was incubated for one minute in western blotting substrate (ECL) at room temperature. The membrane was then put into a protective plastic sheet. Air bubbles and excess substrate solution were removed by

careful smoothing. The membrane was placed in VersaDoc and the exposure time was set to 1min.

Re-incubation with anti-β-actin antibodies re-detection

Membranes after immunodetection with ECL were rinsed with PBS-T two times for five minutes to remove excess ECL and incubated with 10ml mouse anti-β-actin diluted in 5% milk for 0.5 hour at room temperature in a clean plastic box. Afterwards, antibody solution was discarded and a five minutes' washing step with PBS-T was carried out five times on the shaker. Then, 1µl secondary antibody (rabbit anti mouse) was added to 10ml dilute solution (5% skimmed milk powder diluted in PBS-T) and incubated for 0.5 hour at room temperature in the same plastic box. After discarding the secondary antibody solution, a five minutes' washing with PBS-T was performed five times on the shaker. Then the same process of immunodetection with ECL was done as previously described.

Data analysis

Bands were detected using Imaging System 4000 and band optical density was analyzed with Software Quantity One. The results are shown as the ratio of target protein to β-actin in relative optical density (ROD) units.

3.2.7 Statistical analysis

Data were analyzed by using SPSS 20 statistical software. Normal distribution and equal variance (Levene test, $p>0.05$) were performed for all data analyses. To achieve normality, log transformation was used for data that was not normally distributed. To determine the effect of factors such as dG, treatment, GRα-subtype (++, +-, - -), level L1-3, placentome subtype and gender as well as interaction between them on the mean number of GRαBNC, data sets were analyzed using a full factorial model (MANOVA) with these factors. To determine the effect of factors such as dG, treatment and GRα-subtype (++, +-, - -) as well interaction between them on the mean percentage of GRαBNC numbers, data sets were analyzed using MANOVA with these factors. To

determine the effect of factors such as dG, treatment, placentome subtype and gender as well as interaction between them on GRt and GRα protein levels, data sets were analyzed using MANOVA with these factors. When main effects were $p<0.05$ a pairwise comparison (Holm´s Sidak) was performed. Main effects and interactions are shown in the result section as well as in the figure legend when significant ($p<0.05$) and post hoc p-values (Holm-Sidak) are indicated in the figures. The relationship between the mean number of GRαBNCs maternal and fetal cortisol plasma levels of the same cohort of animals as previously reported[87], was determined by correlation analysis (Pearson). Data are presented as mean ± S.E.M. Significant difference was accepted for $p<0.05$.

3.2.8 Housekeeping gene selection

Before determining the reliability of internal control genes using the average expression stability values, we tried preliminary experiments of four internal control genes (18S rRNA; HPRT1; ACTB; RPLPO), which were widely used as housekeeping genes. Since we wanted to investigate the effect of early DEX treatment from early gestation (50dG) to late gestation (140dG), it was important to choose a housekeeping gene with stable expression during the whole gestation. However, none of them remained absolutely unchanged over such a long period and the results were different if we used a single gene or an average of two to three genes as internal control. Recently, a normalization strategy was reported to calculate the minimum number of genes needed to determine a reliable normalization factor and to select the most stable internal control genes in a group of tissues.[134] With this reported method, the designed geNorm VBA applet determines the most stable reference genes from a set of tested genes by calculating the gene expression stability measure M value for a reference gene. A gene expression normalization factor for each tissue sample is calculated according to the geometric mean of a set number of reference genes. Afterwards, depending on the M value, stepwise exclusion is performed to get the final reference genes.

4. Results

4.1 Immunohistochemistry

4.1.1 Localization and distribution

GRt and GRα were localized in the cytosol and nucleus of the trophoblast throughout the placentome and in fetal membranes as described previously.[131] The localization was independent of gestational age, fetal sex, placentome subtype or treatment. GRα staining was also present within the nucleus of BNCs, with three different staining patterns observed: BNCs with two GRα positive stained nuclei (++), BNCs with one positive and one negative stained GRα nuclei (+-) and BNCs lacking any GRα staining in the nuclei (--) (Figure 8 A, B). These three patterns were found in males and females, control and DEX-treated placentomes, independent of placentome subtype or gestational age.

In all fetuses, (++) was the most frequent cell type and (--) the least frequent cell type (MANOVA main effects: dG $p<0.05$, treatment $p<0.05$, GRα-type $p<0.05$; placentome subtype $p<0.05$, level $p<0.05$; interaction: dG*GRα-type $p<0.05$, dG*treatment $p<0.05$, GRα-type*level $p<0.05$, dG*placentome subtype $p<0.05$, dG*level $p<0.05$, placentome subtype*level $p<0.05$, dG*treatment*GRα-type*placentome subtype*level $p=0.05$; Figure 9 A).

The mean number and percentage of (++) in controls significantly increased from 50 to 100dG and decreased from 100 toward 140dG ($p<0.05$), which was more obvious in pregnancies with male fetuses (Figure 9 A, B). Although the mean number of (+-) and (--) did not change across gestation (Figure 9 A), the proportion of total of (+-) and (--) decreased from 50 to 100dG and increased between 100 to 140dG afterwards ($p<0.05$, Figure 9 B). Differences were observed at 50, 100, 125dG in the numbers of different GRα BNC-subtypes at different levels within the placentome (Figure 10).[58] The mean numbers of (++) and (--) were higher in L1 compared to L3 ($p<0.05$;Figure 10 A+B); no sig. level differences at those time points were found for (+-) (Figure 10). At 140dG, the mean number of (++) was lowest in L2 and the mean number of (--) was highest at L2 compared to L1 and 3 (Figure 10 A, C). No specific immunostaining was found for GRβ.

4.1.2 Functional differences in BNC

oPL was present mainly in the cytoplasm of the BNCs without immunostaining in the maternal syncytium as reported previously (Figure 8 D).[58, 59] Colocalistaion with oPL revealed three different staining patterns: BNCs with oPL-positive stained cytoplasm and two GRα-positive stained nuclei (oPL++); BNCs with oPL-positive stained cytoplasm and one positive and one negative GRα stained nucleus (oPL+-) and BNCs without oPL-positive stained cytoplasm and no GRα-stained nucleus (- -) (Figure 8 E).

Caspase-3 localized mainly to the nuclei of BNCs without immunostaining in the maternal syncytium, fetal and maternal stroma. Three different staining patterns were found for caspase-3 in BNCs: BNCs with two caspase-3 positive stained nuclei, BNCs lacking any caspase-3 staining in the nucleus and BNCs with one positive and one negative caspase-3 stained nucleus were found (Figure 8 F0-F2). Double staining in BNCs with GRα and caspase-3 showed more different staining patterns: BNCs with two GRα positive nuclei lacking any caspase-3 staining, BNCs with one positive GRα stained nucleus and one positive caspase-3 stained nucleus, BNCs with two positive caspase-3 stained nuclei lacking any GRα staining, BNCs with one positive GRα stained nucleus and lacking any caspase-3 staining, BNCs with one positive caspase-3 stained nucleus and lacking any GRα staining, BNCs without GRα and caspase-3 staining (Figure 8 G0-G3).

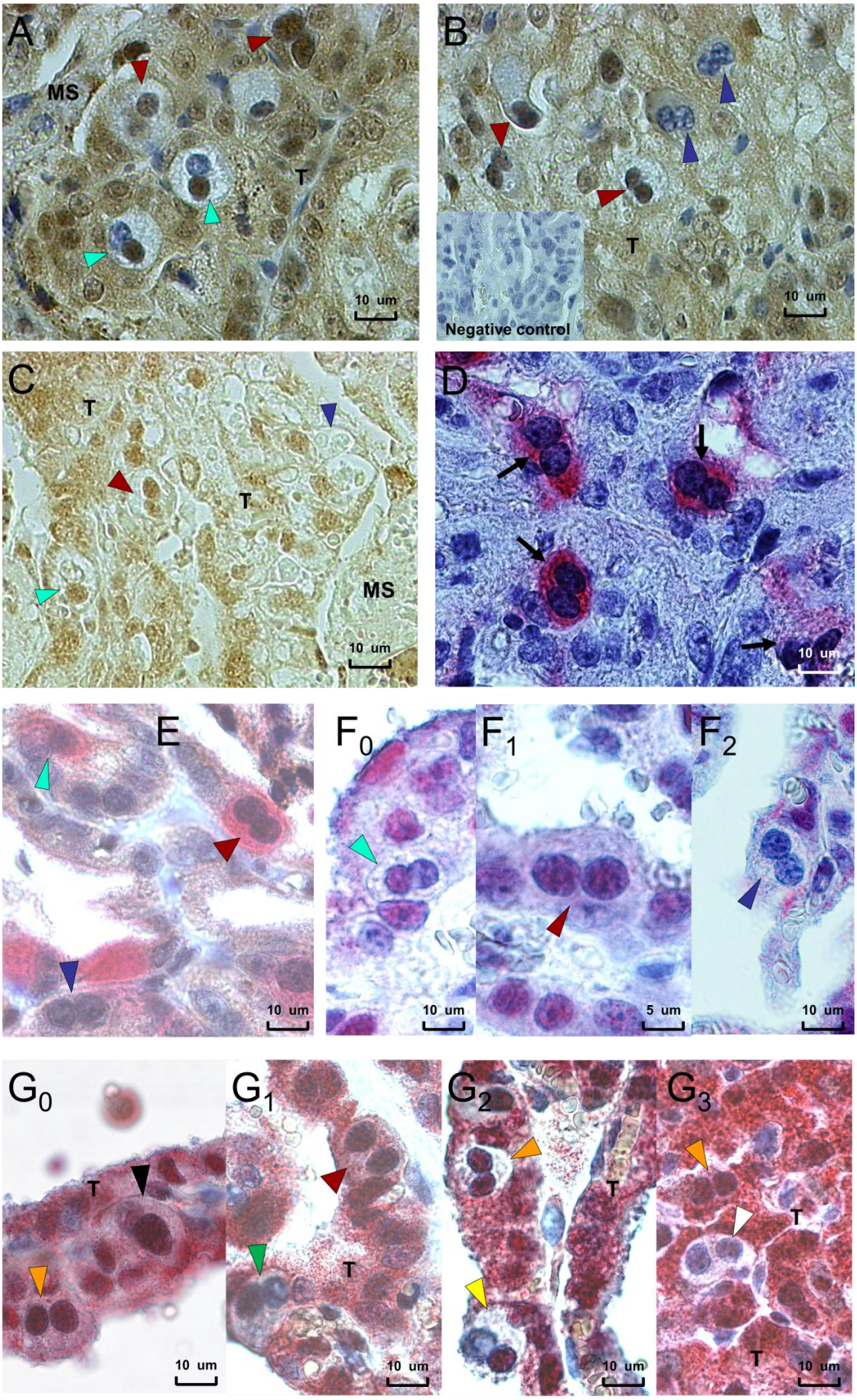

A
MS
T
10 um
B
T
Negative control
10 um
C
T
T
MS
10 um
D
10 um
E
10 um
F0
10 um
F1
5 um
F2
10 um
G0
T
10 um
G1
T
10 um
G2
T
10 um
G3
T
T
10 um

Figure 8: Immunohistochemical localization of GRα, oPL and caspase-3 in BNCs.

A+B) GRα (DAB, brown) Three different nuclear staining patterns of BNCs were found with hematoxylin counterstaining: BNCs with two brown GRα positive stained nuclei (++, red triangles), BNCs with one positive and one negative GRα staining nucleus (+-, light blue triangle), and BNCs lacking any brown GRα staining in the nuclei (--, blue triangles) .

C) GRα (DAB, brown) BNC staining without hematoxylin counterstaining.

D) oPL (Fast Red, red) BNC cytoplasmic staining.

E) GRα (DAB, brown) and oPL (Fast Red, red) BNC double staining: BNCs with oPL positive stained cytoplasm (red) and two GRα positive stained nuclei (brown) marked with a red triangle; BNCs with oPL positive stained cytoplasm (red) and one positive and one negative GRα stained nucleus (one brown one blue) marked with a light blue triangle and BNCs without oPL positive stained cytoplasm and no GRα stained nucleus indicated by blue triangles.

F_0-F_2) Caspase-3 (Fast Red, red) BNC nucleus staining: BNCs with two caspase-3 positive stained nuclei (red) marked with a red triangle, BNCs lacking any caspase-3 staining in the nuclei marked with a blue triangle and BNCs with one positive and one negative caspase-3 staining nucleus(one red one blue) marked with a light blue triangle.

G_0-G_3) Caspase-3 (Fast Red, red) and GRα (DAB, brown) BNC double staining: BNCs with two GRα positive nuclei (brown) lacking any of caspase-3 staining marked with a red triangle, BNCs with one positive GRα stained nucleus (brown) and one positive caspase-3 stained nucleus (red) marked with a black triangle, BNCs with two positive caspase-3 stained nuclei (red) lacking any of GRα staining marked with an orange triangle, BNCs with one positive GRα stained nucleus (brown) and lacking any of caspase-3 staining marked with a green triangle, BNCs with one positive caspase-3 stained nucleus (red) and lacking any of GRα staining marked with a yellow triangle, BNCs without GRα and caspase-3 staining marked with a white triangle. Representative negative control was inserted in B. T=trophoblast, MS=maternal syncytium.[135]

4.1.3 The effect of DEX on GRα localization and distribution

Early DEX treatment sig. decreased the mean number of (++) at 100dG and all three GRαBNC-subtypes at 140dG compared to controls ($p<0.05$; Figure 9 A). The proportion of total of (++) sig. increased from 50 to 100dG and the proportion of total of (+-) and (--) sig. decreased between 50 and 100dG compared to controls (MANOVA main effects: dG $p<0.05$, treatment $p<0.05$, GRα-type $p<0.05$; interactions: dG*GRα-type $p<0.05$, $p<0.05$; dG*treatment $p<0.05$, Figure 9 B). The proportion of all three GRα BNC-subtypes remained the same between 100 and 140dG in DEX group resulting in a sig. lower proportion of (--) and sig. higher proportion of (++) at 140dG as compared to controls ($p<0.05$; Figure 9 B).

The effect of DEX treatment on GRα BNCs numbers varied with different levels of the placentome. At 140dG, DEX sig. reduced the mean number of (++) and (+-) only in L1, while no changes was found in L2 or L3 (Figure 10 A+B). The number of (--) at 140dG was sig. reduced in all 3 levels in DEX groups ($p<0.05$; Figure 10 C).

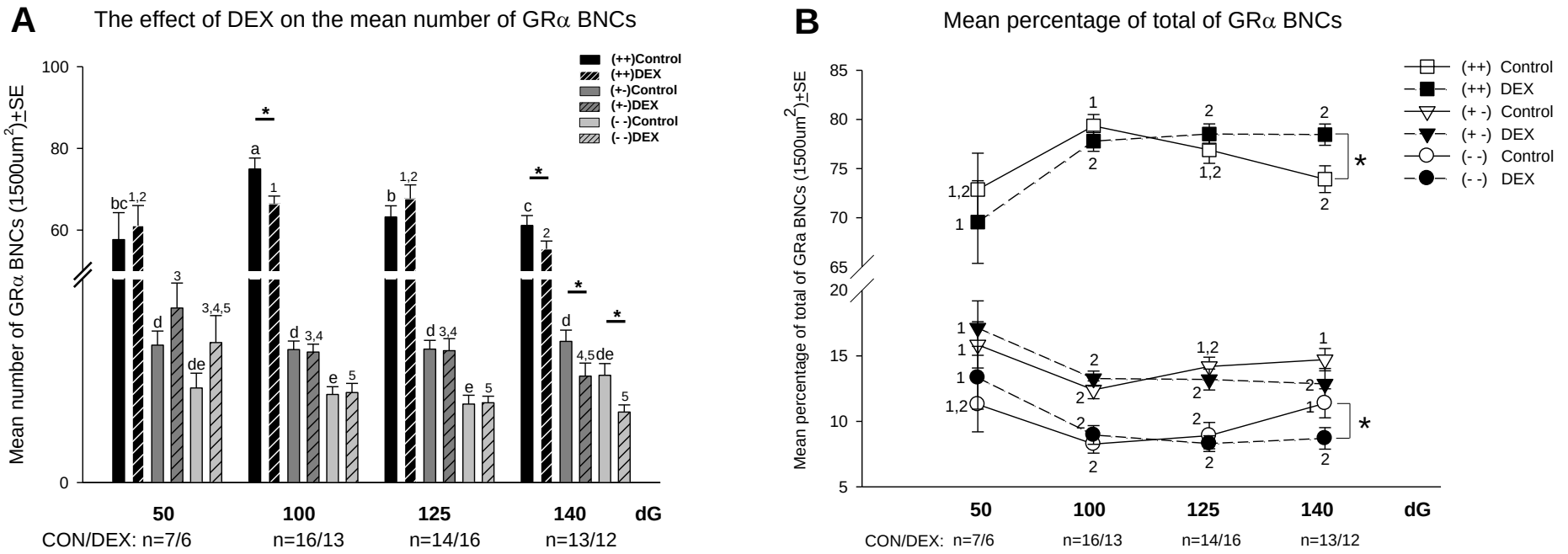
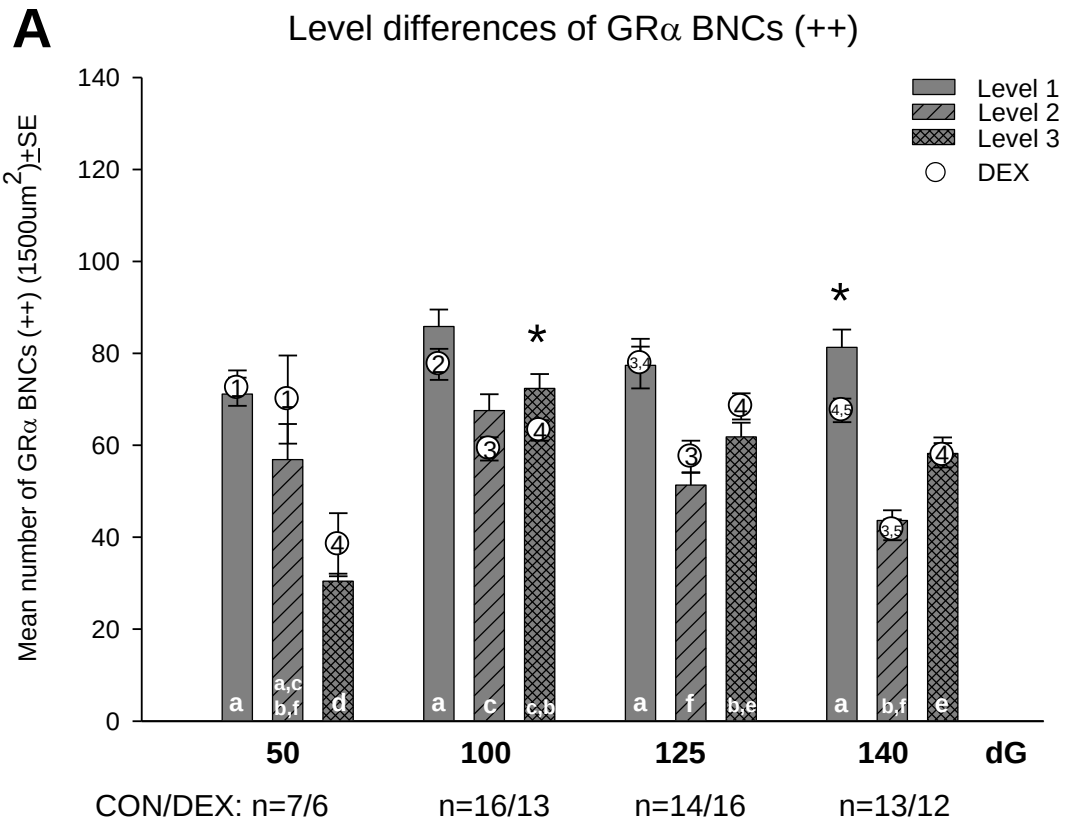
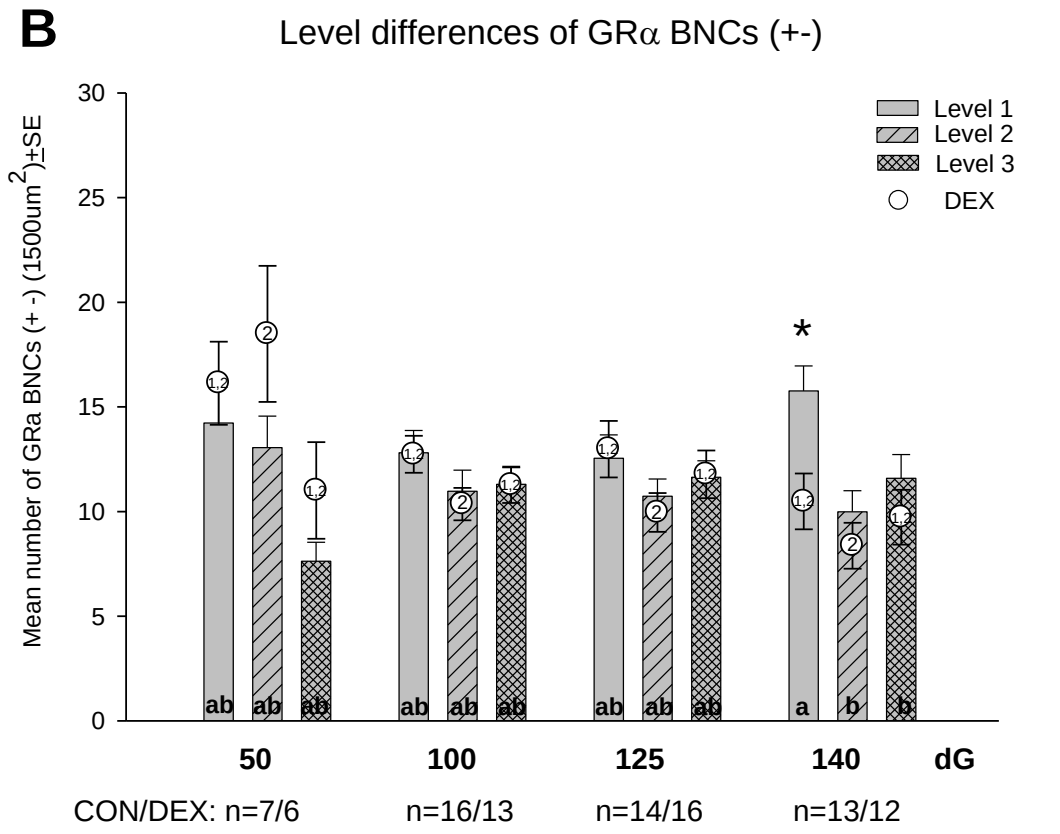


Figure 9 A-B: The effect of DEX on GRα positive stained BNCs.

(A) When main effects were p<0.05, a pairwise comparison (Holm`s Sidak) was performed using a full factorial model (MANOVA) with dG, treatment and GRα-subtype (++, +-, - -) as factors to determine dG, treatment and GRα-subtype (++, +-, - -) effects as well an interaction between them on GRα BNC numbers. MANOVA main effects: dG p<0.05, treatment p<0.05, GRα-type p<0.05; interaction: dG*treatment p<0.05, dG*GRα-type p<0.05. Post hoc p-values (Holm-Sidak) are indicated in figures, sig. difference was accept for p<0.05. Different letters (a–d) indicate sig. differences in control groups across gestation. Numbers show sig. differences in the DEX groups across gestation. Stars represent sig. differences between control and DEX. (B) When main effects were p<0.05, a pairwise comparison (Holm`s Sidak) was performed using a full factorial model (MANOVA) with dG, treatment and GRα-subtype (++, +-, - -) as factors to determine dG, treatment and GRα-subtype (++, +-, - -) effects as well an interaction between them on the mean percentage of GRα BNC numbers. Post hoc p-values (Holm-Sidak) are indicated in figures. Different numbers represent sig. differences across gestation per subtype. Stars represent sig. differences between control and DEX per subtype. The number of sheep included in the study are given below (n=Control/DEX). Sig. difference was accept for p<0.05.[135]


A
Level differences of GRα BNCs (++)
Mean number of GRα BNCs (++) (1500um2)±SE
Level 1
Level 2
Level 3
DEX
140
120
100
80
60
40
20
0
a
a,c
b,f
d
a
c
c,b
a
f
b,e
a
b,f
e
*
*
50
100
125
140
dG
CON/DEX: n=7/6
n=16/13
n=14/16
n=13/12


B
Level differences of GRα BNCs (+-)
Mean number of GRa BNCs (+ -) (1500um2)±SE
Level 1
Level 2
Level 3
DEX
30
25
20
15
10
5
0
ab
ab
ab
ab
ab
ab
ab
ab
ab
a
b
b
*
50
100
125
140
dG
CON/DEX: n=7/6
n=16/13
n=14/16
n=13/12

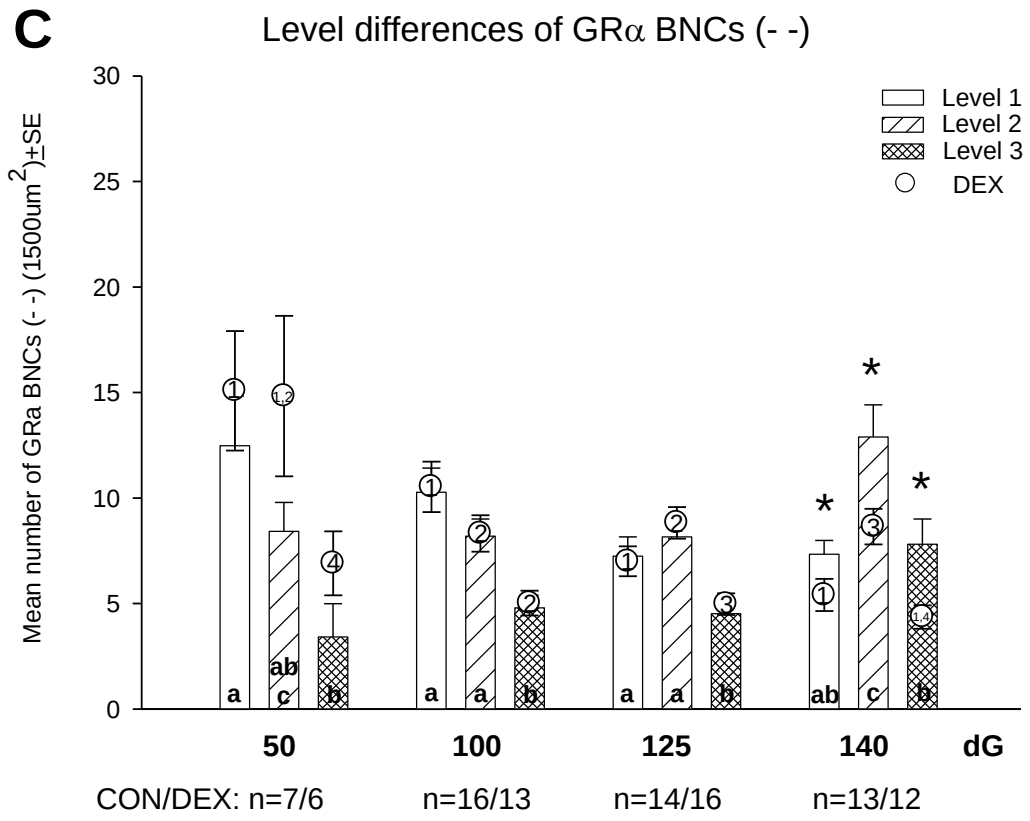


Figure 10 A-C: The effect of DEX on the mean number of GRα BNCs in Level 1-3

(A) The effect of DEX on the mean number of (++). (B) The effect of DEX on the mean number of (+-). (C) The effect of DEX on the mean number of (--). Mean data was analyzed by MANOVA with dG, levels and treatment as factors. When main effects were $p<0.05$, a pairwise comparison (Holm`s Sidak) was performed. MANOVA main effects: dG $p<0.05$, treatment $p<0.05$, GRα-subtype $p<0.05$; level $p<0.05$, placentome subtype $p<0.05$; interaction: dG*treatment $p<0.05$, dG*GRα-subtype $p<0.05$, GRα-type*level $p<0.05$, dG*placentome subtype $p<0.05$, dG*level $p<0.05$, placentome subtype*level $p<0.05$, dG*treatment*GRα-subtype*placentome subtype*level $p=0.05$. Post hoc p-values (Holm-Sidak) are shown in figures and sig. difference was accepted for $p<0.05$. Sig. differences in CON group across gestation are indicated by letters (a–d). Sig. differences in the DEX group across gestation are indicated by numbers. Sig. differences between CON and DEX groups are indicated by stars. The sheep number is given under the column (n=CON/DEX). D) Number of BNCs in 18 random fields of view grouped into three levels was counted. Two sections were analyzed for each sample. A total over 6,000 pictures were included.[135]

4.2 Placental glucocorticoid receptor protein levels and the effect of DEX

GRt protein was analyzed using western blot and a single 97 kDa band was found as described previously.[59] The single band was proven to be specific for GRt by protein sequencing (GenBank: EU371026.1).

In control males, GRt protein levels were highest at 125dG and thereafter decreased towards term (MANOVA main effects: dG $p<0.05$; interaction: treatment*type $p<0.05$, dG*treatment*sex $p<0.05$; Figure 11 A). No significant change in GRt protein levels was observed in females across gestation. In males, DEX sig. decreased GRt protein levels at 125dG (predominantly in C- and D-subtypes; Figure 11 A), while in females DEX sig. increased GRt protein levels at 50dG (predominantly in A-subtypes) and at 125dG (predominantly in B-subtypes; Figure 11 A).

GRα protein was detected at 95 kDa as described previously[78, 131] and was proven to be specific with a blocking peptide (Figure 11 A). In both males and females, GRα protein levels were lowest at 140dG (MANOVA main effects: dG $p<0.05$; interaction $p>0.05$; Figure 11 B). DEX did not affect GRα protein level, although in males and females, GRα protein levels tended to decrease at 50dG after DEX treatment ($p>0.05$, Figure 11 B). GRα protein levels did not change with different placentome subtypes.

The band found at 97kDa and previously described as GRβ[78] could not be blocked with the specific blocking peptide that is currently commercially available (Figure 11 B).

4.3 Correlation analyses

In control females, the number of GRα (++) BNC was inversely correlated with previously[87] reported fetal cortisol levels of the same cohort of animals ($r=-0.446$, $p<0.05$), which was not seen in the DEX group. In males, fetal cortisol levels were inversely correlated with GRα protein levels in DEX groups ($r=-0.487$, $p>0.05$). None of GRt protein levels or GRα protein levels was sig. correlated with maternal or fetal cortisol and ACTH plasma levels.

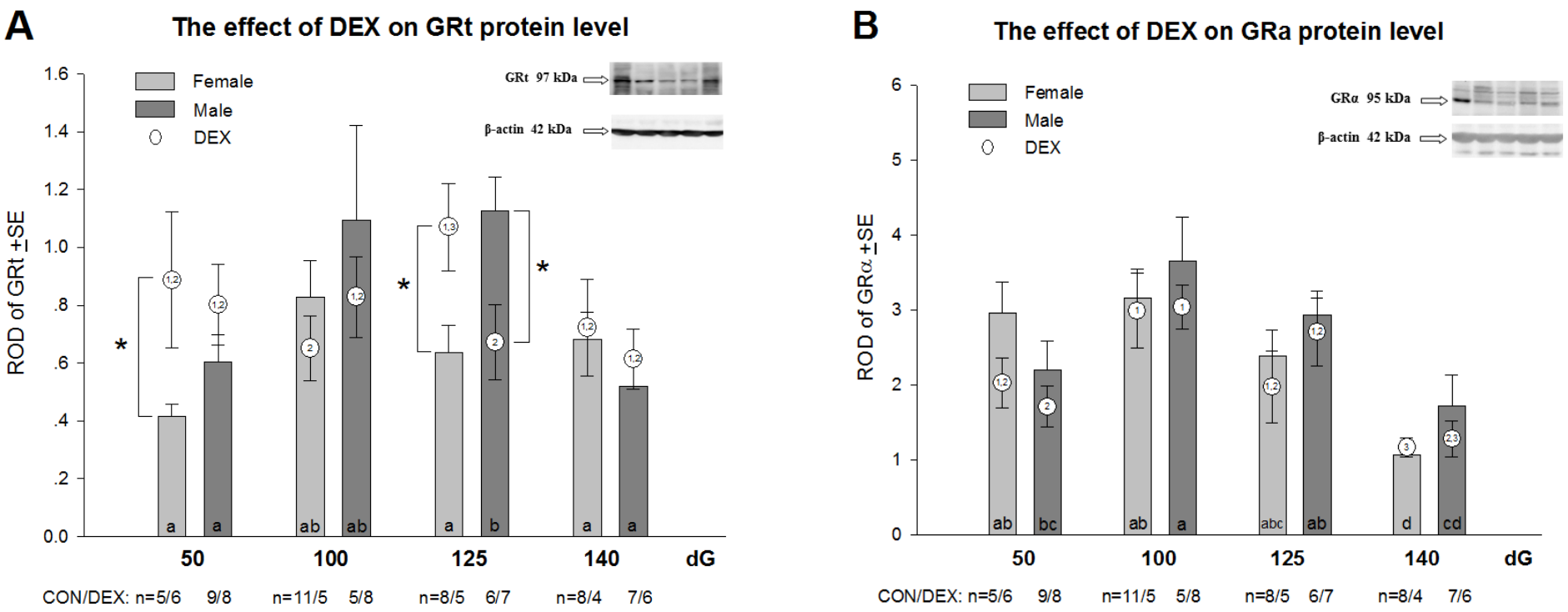


Figure 11 A-B: The effect of DEX on GRt (A) and GRα (B) protein level in males and females.

Mean data was analyzed by MANOVA with dG, sex and treatment as factors. When main effects were $p<0.05$, a pairwise comparison (Holm`s Sidak) was performed. MANOVA main effects GRt: dG $p<0.05$, interaction: Treatment*type $p<0.05$, dG*treatment*sex $p<0.05$. MANOVA main effects GRα: dG $p<0.001$, interaction: $p>0.05$. Post hoc p-values (Holm-Sidak) are shown in figures. Sig. differences in CON group across gestation are indicated by letters (a–b). Sig. differences in the DEX group across gestation are indicated by numbers. Sig. differences between CON and DEX groups are indicated by stars. The sheep number is given below. ROD was calculated as (GRα/β-actin)/internal control. Sig. difference was accept for $p<0.05$.[135]

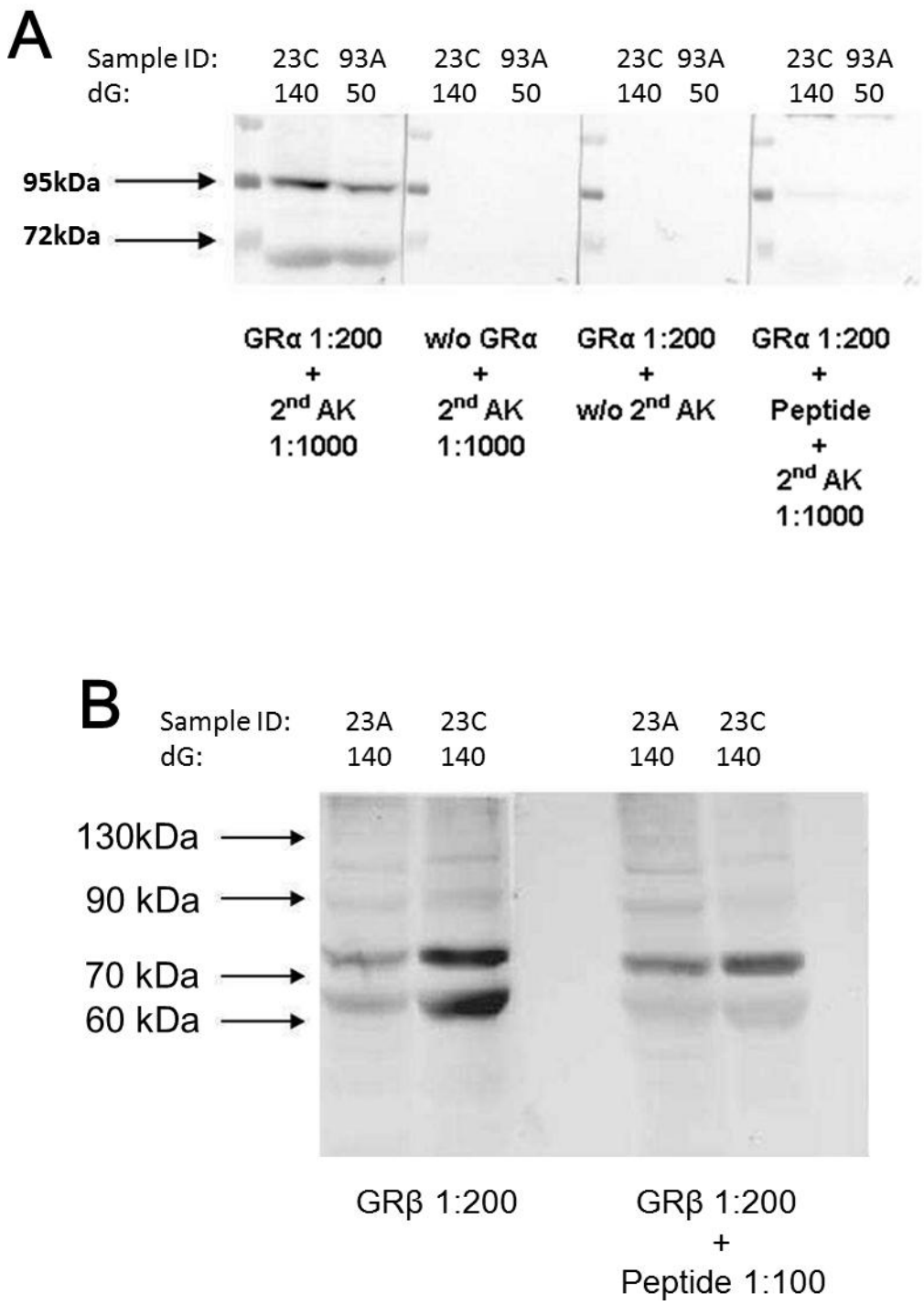


Figure 12 A-B: Western blot membranes of GRα and GRβ incubated with blocking peptide.
A) GRα protein was identified at 95 kDa and was undetectable if the membrane was incubated with blocking peptide during the experiment. B) We were unable to find a specific band for GRβ. The band found at 97kDa was still visible if the membrane was incubated with the specific blocking peptide during the experiment.[135]

5. Discussion

DEX treatment in early pregnancy exhibited a transient and sex-specific effect on placental and fetal development. Three GRα BNC phenotypes (++, +-, --) were identified, suggesting different functional activity. DEX administration significantly increased the placental GRt protein level in females at 50 and 125dG, but decreased placental GRt protein level in males at 125dG as compared to controls. GRα protein levels were not changed after DEX administration. Effects were sex- and cell-type-dependent, indicating that the responsiveness of the placenta to endogenous cortisol was modified by DEX treatment.

5.1 The effect of DEX on GR in the ovine placenta

Exogenous GC exposure in pregnancy may have potentially long term impact on health of the affected individual in later life (perinatal programming).[65] To investigate the underlying mechanisms, our current study focused on the presence of placental GR, which may play an important role in mediating the regulation of BNC function and placental development after DEX treatment in early pregnancy.

Temporal and tissue-specific expression of GRt, GRα and GRβ proteins in ovine intrauterine tissues during the onset and progression of spontaneous labor have been demonstrated previously.[131] GRt, GRα, and GRβ proteins were localized to trophoblast cells of the placenta, fetal amnion, chorion and maternal endometrium.[131] Although the localization did not change during labor progression, the protein levels of GRt and GRα increased in placental tissues during labor, indicating increased responsiveness of placenta to cortisol at the onset and during progression of labor.[131]

The effects of cortisol treatment on GR vary according to previous reports due to different treatment protocols or cell types.[112, 136, 137] For example, in Hela S3 cells, both GR mRNA and protein level were significantly decreased after DEX treatment for 24h, 48h and 2 weeks after treatment, which was reversible upon DEX removal.[113] Cells

chronically treated with DEX (for up to 2 years) had no measurable GR mRNA or protein levels and did not recover after removal,[113] suggesting different responsiveness of GR to short-term or long-term DEX treatment. Previous studies in sheep placenta showed that direct cortisol infusion to the fetus resulted in increased GRα but not GRt protein levels.[131] Moreover, the expression of GRt and GRα did not change significantly in human placenta after BET treatment before term compared with placentas from normal pregnancies.[114]

Gupta et al. reported staining of GRβ, localized to the cytosol of trophoblast cells, in amnion epithelial cells, chorionic epithelium of the fetal membranes and in maternal endometrial epithelium.[131] Although we used the same antibody and tried different protocols, we were unable to localize specific GRβ staining within the placentome after testing with a newly available blocking peptide and were also unable to detect a specific GRβ protein band, as it was reported previously.[78] We and others speculate that the expression of GRβ is either very low in sheep placenta or the GRβ antibody reported by other authors revealed a non-specific binding of GRβ.[78]

Previously, it was suggested that the GR is formed from a single gene, but the notion of a "one gene-one receptor" pattern has been changed by findings according to which various functionally different GR-isoforms are generated from alternative splicing.[99, 138] Additionally, studies revealed that the human proteome expands to a much larger size than the encoding genome of 25,000 genes, suggesting that different receptor isoforms are variously modified after translation.[139] Accordingly, the expressed complement and composite actions of the individual GR isoforms play an important role in the sensitivity and specificity of the glucocorticoid response element. In our study, GRα protein levels were not changed after early DEX treatment, but were significantly correlated with oPL protein (males+females) and fetal cortisol levels (males), suggesting a greater importance of GRα for cortisol responsiveness and oPL production/secretion. Direct cortisol infusion to the fetus increased the protein level of GRα but not GRt in sheep placenta,[131] which suggests that additional GR isoforms may be involved in to balancing

the GR total protein level. Recently, Saif et al. found 12 isoforms of the GR in the human placenta, which were localised to the trophoblast.[140] Expression of these isoforms varied according to cellular location in either the cytoplasm or the nucleus, fetal sex, fetal size, but not all isoforms were expressed in every individual.[140] In our study, GRt protein levels were not associated with placental oPL levels or fetal and maternal plasma oPL levels as previously reported.[128] GRα protein levels were stable across gestation and were not affected by early DEX treatment. This may indicate that other GR isoforms, and not only GRα, may also play a critical role in regulating the level of oPL, or the effect of DEX resulted in altered GRt protein level but not GRα protein level, although GRα was considered to be the active form of GR. Therefore, we would like to suggest that other GR isoforms may also exist in the sheep placenta and contribute to the changes observed in GRt protein but not GRα protein, which needs further investigation.

BNCs can be divided into two groups according to their locations: the cotyledonary BNCs and the intercotyledonary BNCs,[62] and functional differences have been implied previously: the cotyledonary BNCs metabolize more pregnenolone than the intercotyledonary BNCs and generate a narrower range of minor metabolites than the intercotyledonary BNCs.[62] Now, we were able for the first time to demonstrate three different immunostaining patterns of GRα in BNCs: (++) GRα BNCs, with two GRα positive stained nuclei, being the most frequently observed staining pattern; (--) GRα BNCs, lacking any of GRα nuclear staining, being the least frequently observed staining pattern; (+-) GRα BNCs, with one GRα positive stained nucleus and one GRα negative stained nucleus, being the intermediate frequently observed staining pattern.

However, the separate function and the developing trajectory of these three BNCs types still remain unclear. Since BNCs produce oPL, co-localization of GRα and oPL was used to investigate the difference among these three GRα BNC types regarding the oPL-granules in the cytoplasm. Indeed, (++) GRα BNCs and (+-) GRα BNCs stained positively for oPL in the cytoplasm, whereas the (--) GRα BNCs did not have positive oPL staining in the cytoplasm. Based on our current and previous observations and the

frequency of occurrence, we hypothesized that (++) GRα BNCs is a potential “active” BNC form; (--) GRα BNCs is an “immature” or “inactive” BNC form and that (+-) GRα BNCs is an “intermediate” BNC form (Figure 13).

Apoptosis plays an important role in maintaining balance between the clearance of non-functional cells and the formation of new cells in different organs, such as placenta, where apoptosis was also proved to be crucial in the normal development, remodeling and aging.[141] Additionally, it has been demonstrated that apoptosis increases during gestation, suggesting that apoptosis is a normal physiological phenomenon throughout gestation[142] and thus the regulation of apoptosis is important in the placental development, differentiation and function.[143] An imbalance of this process can lead to severe pathologies such as intrauterine growth retardation. This is supported by studies that reported a significantly higher incidence of apoptosis in IUGR placenta compared with controls.[144]

Also, GC treatment can be associated with increased rates of apoptosis.[128, 145-148] It has been reported that BET and DEX treatment for more than 96 hours caused apoptotic cell death in nasal polyp fibroblasts determined by agarose gel electrophoresis.[147] Due to increased apoptosis in chondrocytes, a time-dependent decrease in chondrocyte viability was also found after GC administration.[146] In the rat placenta, as in the case of DEX treatment, carbenoxolone elevated endogenous GC levels by reduction of 11β-HSD2, also causing increased apoptosis, parallel with decreased fetal and placental weights.[149] This was also supported in a study in the rat where GC treatment not only caused IUGR but also reduced placental weight by almost 50% between 13 and 20dG, which was consistent with increased apoptosis.[145]

It has been shown previously in our own DEX treatment model in sheep that DEX administration early in pregnancy significantly increased the placental mRNA expression levels of pro-apoptotic (caspase-3 at 100dG, Bax and p53 at 125dG) markers and significantly decreased anti-apoptotic markers (PCNA at 100dG), which suggests that DEX induces apoptosis via the intrinsic pathway.[128, 145] Consistent with this,

co-localisation of GRα with active caspase-3 revealed strong nuclear caspase-3 staining in the (--) GRα BNC, whereas in the (++) GRα BNC, no caspase-3 nuclear staining was observed, which supports our hypothesis that the (--) GRα BNC and the (++) GRα BNC may be “inactive” and “active” form, respectively.

This study was not originally designed to study the underlying mechanisms of the formation of different BNC types. However, in view of the fact that the proportion of (++, +- and --) GRα BNCs changed with advancing gestational age, we hypothesize that these changes are associated with placental maturation in early gestation and with fetal HPA maturation in middle and late gestation. So far, evidence suggested, but not definitely proved, that BNCs develop from the division of a single uninucleate trophoblast cell in which the nuclei divide but not the cytoplasm.[44-46] However, in a recent paper, BNC formation in the human brain due to multiple sclerosis lesions was demonstrated by heterotopic cell fusion of two uninucleate cells without subsequent chromosomal loss.[150] We hypothesize that the different GRα-staining patterns represent different maturational stages and functions of a single BNC.

Although the regulatory factors regulating these processes are unknown and could not be investigated in this study, we may propose that:
1.) Cortisol itself may play a vital role in mediating these events;
2.) The production and migration of BNCs itself may be involved in ruminant implantation and placental growth.

In early gestation (50 to 100dG) we observed that the proportion of (++) increased and the proportion of (+-)/(--) decreased, which may be explained by the naturally occurring maturation of placenta and BNCs. However, in middle and late gestation (100 to 140dG), as the fetal HPA axis matures and begins to secrete higher concentrations of cortisol, a decrease in the proportion of (++) GRα BNCs and an increase in the proportion of (+-)/(--) GRα BNCs were observed. This is in agreement with previous reports on the effect of normal surge of GC late in gestation and is consistent with the results that fetal

hypophysectomy or fetal adrenalectomy prevented this normal prenatal decline in BNC numbers.[59] Therefore, early in pregnancy, BNC may develop directly from “premature” (--) GRα BNC to “activated” (++) GRα BNCs, since the number of “intermediate” (+-) GRα BNC did not change. Later in pregnancy, under the increased exposure to fetal cortisol due to HPA axis activation, BNC may develop from “premature” (--) GRα BNC to “active” (++) GRα BNC via the “intermediate” (+-) GRα BNC, since an increased number of “intermediate” (+-) GRα BNC was found during this period (Figure 13).

We have previously shown that BET treatment late in gestation significantly decreased birth and placenta weights and was associated with reduced mean number of BNCs, oPL protein concentration and fetal and maternal plasma oPL concentration in sheep.[58] Recently we could show that early DEX treatment in sheep resulted in a reduction in the mean number of BNCs and placenta oPL protein levels, but DEX did not influence fetal or maternal plasma oPL levels.[128] We hypothesized that plasma oPL level were not only influenced by the raw number of BNCs but also by the distribution and presence of activated vs. inactivated BNCs and that other regulatory mechanism may be involved.

DEX treatment early in gestation increased fetal adrenal activation and fetal adrenal steroidogenic activity near term and resulted in an altered fetal HPA axis.[87] In this study, early DEX administration significantly decreased the number of (++) GRα BNCs at 100 and 140dG compared to controls and at the same time changed the proportion of three GRα BNC types resulting in a relative decrease in the percentage of (+-)/(--) GRα BNCs and an increase in the percentage of (++) GRα BNCs. This suggests that early DEX treatment may reduce the sensitivity of BNCs to endogenous cortisol near term and thereby impede the conversion from (++) GRα BNCs to (+-)/(--) GRα BNCs.

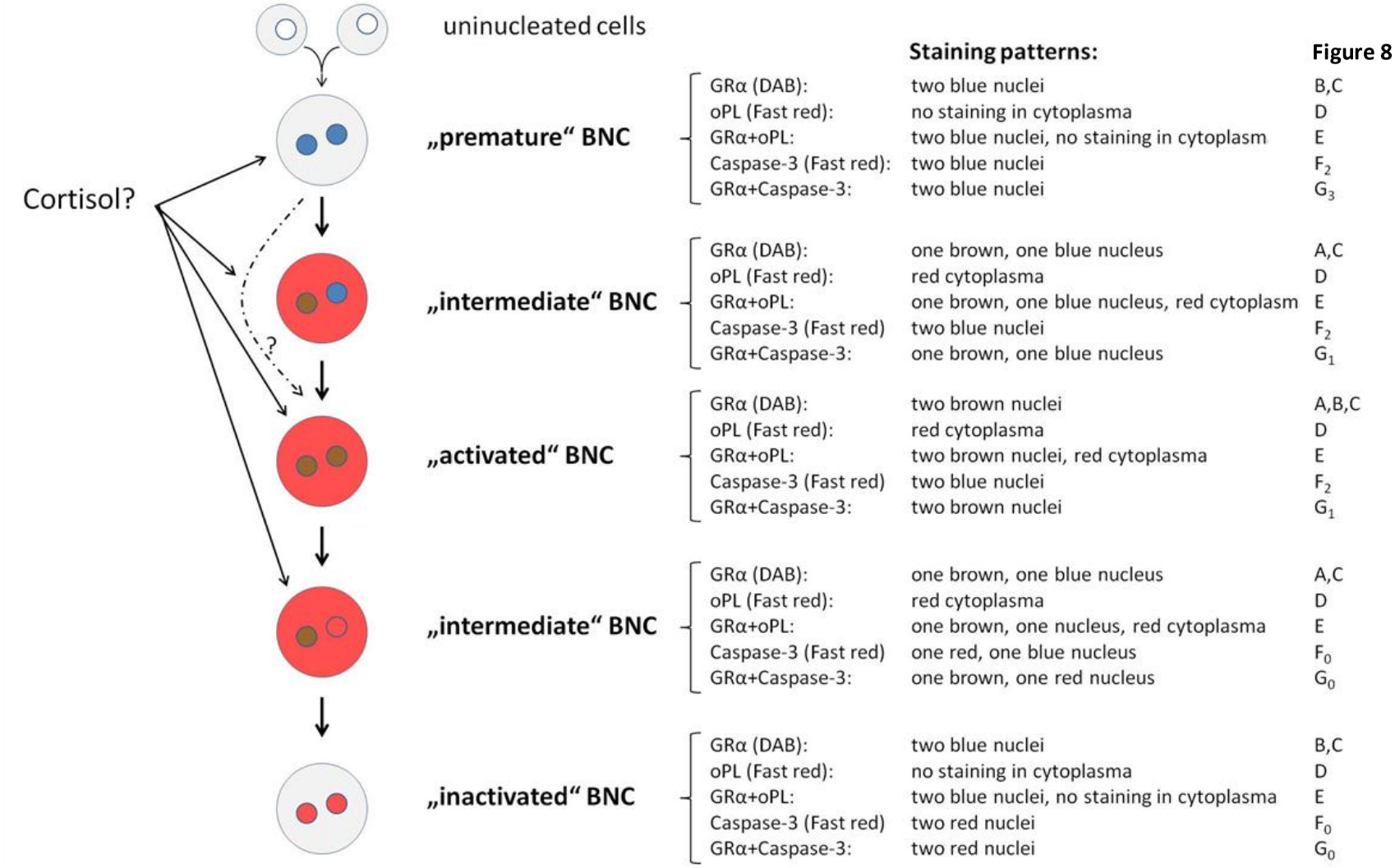


Figure 13: Hypothesis for the formation of three GRαBNC-subtypes in sheep placenta.

Different BNC “activity stages” are shown and analyzed via the immunohistochemical localization of GRα, oPL and caspase-3 and the co-localization of two of them in BNCs in sheep placentomes. The development trajectory may differ according to the period of pregnancy: early in pregnancy, premature (--) GRα BNCs may develop directly to activated (++) GRα BNCs, supported by the unchanged number of intermediate (+-)GRα BNCs; later in pregnancy, due to increased fetal cortisol due to HPA axis activation, premature (--) GRα BNCs may develop to the intermediate (+-) GRα BNCs and further to the activated (++) GRα BNCs, which is supported by an increase in the number of intermediate (+-) GRα BNCs.

5.2 Influence of placentome subtypes

A morphologic classification system for placentomes in sheep placenta was first described by Vatnick.[50] It was reported that fetal weight was decreased by maternal under-nutrition in early gestation, which could be compensated by an increase in the proportions of C and D placentome subtypes in the uterus.[151] Therefore, C and D placentome subtypes were suggested to be more effective than type A and B placentomes in nutrient transportation[151] and thus more nutrients may be transferred by the increased conversion from A and B placentomes to more functional C and D types.

Although some previous studies have suggested that the proportion of D-subtypes increased with gestation process in control pregnancies,[55, 152] Braun et al. found that A-subtype was the predominant subtype and the B-subtype was the second-most popular subtype in control pregnancies and did not alter in number between early and late gestation.[39] D-subtypes did not show any significant increase over the course of gestation.[39]

In the present study we found differences with respect to placentome type. Compared to controls, DEX significantly increased GRt protein levels in females at 50 dG and at 125 dG (Figure 11). This change predominantly came from A-subtypes at 50 dG and B-subtypes at 125 dG ($p<0.05$). In males however, GRt protein levels were significantly decreased at 125 dG compared to controls (Figure 11), mainly in C- and D subtypes. Moreover, it was found that the number of GRα BNCs decreased at 100dG in A-subtypes, at 125dG in B-subtypes and at 140dG in C-subtypes.

Based on previously reports and the present study results, we hypothesize that A and B-subtypes were more easily affected by GC in early and mid gestation, maintaining the basic function of placenta, whereas C and D-subtypes, which are more easily affected by GC in late gestation, may be play a greater role in the adaptive response of the placenta to external influences late in gestation.

5.3 The influence of placentome level

It has been shown that protein secretion of BNCs was different between cotyledonary and intercotyledonary BNC populations and suggested that protein expression is dependent on the anatomical localization of BNCs.[62] Thus we divided the placentome into 3 levels as described above (Figure 7) to explore whether there is a level-dependent effect of DEX on the number of BNCs and the role of anatomical localization of BNCs.

It has been reported that the distribution of BNCs at middle pregnancy was similar among the three levels in controls, whereas the mean number of BNCs in controls were highest in L1 when compared with L3 near term ($p<0.001$).[58] In this study, we found that the mean number of GRα BNCs were highest in L1 compared with L3 across gestation. Additionally, the mean number of GRα (++) BNCs in L2 and L3 decreased ($p<0.05$), whereas the mean number of GRα (--) BNCs increased in L2 ($p<0.05$) and L3 between 100 and 140dG (Figure 10). In previous studies only BNCs positively stained for oPL were counted as a marker for functional BNCs, whereas in the present study, all BNCs positively but also negatively stained for GRα were counted, thus indicating that the mean number of all BNCs and not only the “active” and positively stained by oPL were evaluated.

It was previously demonstrated that the placental BNC population is continually developing parallel with the expanding villous tissue.[58] BET treatment quickly reduced the mean number of BNCs in L2 and L3, which suggests that new BNCs were generated in L1 and then migrate to L2 and L3 with the continuously developing terminal villous.[58] Consistent with this, in the present study, the mean number of (++) was highest in L1 compared to L3. However, early DEX treatment significantly reduced the number of (++) and (+-) in late pregnancy (at 140dG) in L1 compared to controls, indicating either a decreased production of BNCs or an increased migration and infusion of BNCs after DEX administration. To reveal the functional difference of BNCs between the 3 levels of the placentome, further investigations need to be done.

5.4 Sex-specific effects

Exogenous GC exposure in pregnancy can result in sex-specific changes in fetal growth, associated with structural and functional changes in the placenta. Sex-specific alterations to a changed environment in utero have been described both in animal and in human studies.[6, 81, 121-127] It has been reported that, as a model for maternal distress, early DEX treatment resulted in a sex-specific, transient decrease in fetal weight and crown rump length at 100dG only in female fetuses, associated with significantly lower BNC numbers and increased apoptotic markers.[87, 88, 128] It has also been found that GC treatment late in gestation resulted in sex-specific changes of mean number and proportion of placentomes.[39] By observation of 12 isoforms of GR, Saif et al. found a sex difference in cortisol-regulated pathways of the placenta and a difference in birthweight outcomes, associated with cord blood cortisol levels that indicated that males seemed to be more glucocorticoid resistant, whereas females were more sensitive to GC.[140]

In the present study we were able to analyze GRt and GRα protein levels from early pregnancy (50dG) to late pregnancy (140dG) for females and males separately. Sex-specific changes were found regarding GRt protein levels: in females, no significant changes were found in GRt protein levels across gestation, whereas in males, highest GRt protein levels were found at 125dG, which decreased towards 140dG. In both females and males, placental GRα protein levels were lowest at 140dG, indicating a reduced placental responsiveness to cortisol near term.

Early DEX treatment significantly increased GRt protein levels at 50dG, mainly in female fetuses. Interestingly, GRt protein levels were significantly decreased in males but increased in females at 125dG. While a constant placental GC sensitivity is maintained in female fetuses in terms of a preferential survival strategy for ensuring reproductive capacity and species conservation, the placenta becomes temporarily GC-resistant in male fetuses. Our result was consistent with previous studies and the possible mechanism for this sex-specific difference may be associated with a translocation of GR-A and GR-P to the nucleus, resulting in a GC-resistance in males, whereas the activation of GRα in females was associated to a higher cortisol sensitivity.[140]

6. Conclusions

Exogenous doses of maternal glucocorticoids (GC) in pregnancy are associated with sex-specific fetal growth inhibition and structural and functional changes in the placenta, which potentially can have life-long impact on the health of the affected individual (fetal programming). Sex-specific strategies for adapting to a changed environment in utero have been described both in animal and in human studies.

To date, this study for the first time demonstrated the presence of three different GRα-BNC-subtypes in sheep placenta. The number of GRα BNCs was regulated by early DEX treatment and this effect was long lasting and sex-, subtype-, placentome-subtype- and level-dependent. Early in pregnancy, DEX may reduce the function of BNCs via increased proportion of “inactive” BNC compared to controls. Apoptosis may play an important role in the conversion among the three BNC-subtypes. This is supported by co-localisation studies with caspase-3, oPL and GRα. Late in pregnancy, early DEX treatment resulted in an increased mean percentage of “active” BNC and decreased the mean percentage of “inactive/intermediate” BNC compared to controls, suggesting that early DEX treatment possibly reduces the sensitivity of GRα in BNCs to the natural rise of fetal cortisol levels near term.

Studies in sheep have shown that early maternal dexamethasone (DEX) therapy, as a model for maternal distress, did not lead to growth inhibition in male fetuses, whereas in female fetuses DEX treatment resulted in a transient growth reduction. In females, adaptation strategies to DEX treatment were observed in particular with respect to the distribution and function of the placentomes, the fetal HPA axis activity and postnatal stress reactivity. While in female fetuses a constant placental GC sensitivity is maintained, possibly in terms of a preferential survival strategy for ensuring reproductive capacity and species conservation, it seems that in male fetuses, due to increased GC exposure, the placenta becomes at least temporarily GC-resistant. Our understanding how endogenous GC and/or overexposure to exogenous GC can influence fetal and placental development begins with the glucocorticoid receptor (GR). More than 8 different GR-isoforms have been described and different splice variants are suspected to

have a different biological activity or responsiveness to physiological stimuli. We suspect that the sex-specific sensitivity to GC is related to a different GR distribution, expression and/or interaction of GRα as transduction stimulating GR vs. other placental GR isoforms and that maternal DEX exposure influences those parameters. Therefore, we propose studies in sheep (ovis aries) with the objective of sex- and placentome-specific identification and localization of placental GR-isoforms and the evaluation of the physiological function of the GR- isoforms for the fetal and placental development as a possible mechanism of gender-specific GC resistance.

Thus, laser capture microdissection for example could be used to isolate single GRα BNC and divide them according to the double staining with anti-GRα. Functional differences and maturational stages could be identified directly by measuring mRNA content of these cells. GR isoforms could be identified by Western Blot and valuable information on the role of GR for the sex-specific adaptation strategies of fetal programming and paradigmatic insights into the mechanisms, how in populations and individuals 'programming errors' may develop, are expected.

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9. Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection

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10. Publications

Posters:

The 13th World Congress on Controversies in Obstetrics Gynecology & Infertility 2010
"Early dexamethasone administration decreased the number of binucleate cells in sheep placenta." Wenbin Meng, **Hongkai Shang**, Deborah M Sloboda, Shaofu Li, Andreas Plagemann, Joachim W Dudenhausen, John P Newnham, John RG Challis, Thorsten Braun. Controversies in obstetrics, gynecology and infertility. 2010 Nov.4

7th World Congress on Developmental Origins of Health and Disease 2011
"Ontogeny of glucocorticoid receptor alpha positive stained binucleate cells in sheep placenta." **Hongkai Shang**, Wenbin Meng, Deborah M. Sloboda, Shaofu Li, Andreas Plagemann, Joachim W Dudenhausen, John P Newnham, John RG Challis, Thorsten Braun. JDOHAD; 2011. p. PI-096

7th World Congress on Developmental Origins of Health and Disease 2011
"Long term effects of early Dexamethasone treatment on glucocorticoid receptor alpha and the regulation of binucleate cell function in sheep placenta." **Hongkai Shang**, Wenbin Meng, Deborah M. Sloboda, Shaofu Li, Andreas Plagemann, Joachim W Dudenhausen, John P Newnham, John RG Challis, Thorsten Braun. Developmental Origins of Health and Disease; 2011. p. PI-370.

IFPA 2011
"Early Dexamethasone (DEX) exposure has lasting effects on glucocorticoid receptor alpha (GRa) in sheep placentomes." Thorsten Braun, **Hongkai Shang**, Wenbin Meng, Deborah M Sloboda, Shaofu Li, Andreas Plagemann, Joachim W Dudenhausen, John P Newnham, John RG Challis; Placenta, Volume 32, Issue 9, A72

IFPA 2013
"Sex-specific differences in the sheep placenta." Thorsten Braun, Wenbin Meng, **Hongkai Shang**, Shaofu Li, Deborah M Sloboda, Loreen Ehrlich, Huaisheng Xu, Wolfgang Henrich, Joachim W Dudenhausen, Andreas Plagemann, John P Newnham and John RG Challis; Placenta Volume 34, Issue 9, Page A91

IFPA 2013
"Early maternal dexamethasone administration and the effect on ovine placental lactogen." Thorsten Braun, Wenbin Meng, **Hongkai Shang**, Shaofu Li, Deborah M Sloboda, Loreen Ehrlich, Huaisheng Xu, Wolfgang Henrich, Joachim W Dudenhausen, Andreas Plagemann, John P Newnham and John RG Challis; Placenta Volume 34, Issue 9, Page A92

Manuscripts:

Paper: "Effects of maternal Dexamethasone treatment early in pregnancy on glucocorticoid receptors in the ovine placenta." **Hongkai Shang,** Wenbin Meng,

Deborah M Sloboda, Shaofu Li, Loreen Ehrlich, Andreas Plagemann, Joachim W Dudenhausen, Wolfgang Henrich, John P Newnham, John R Challis, Thorsten Braun. Reprod Sci. 2014 (ahead of print).

Paper: “Early dexamethasone treatment induces placental apoptosis in sheep.” Thorsten Braun, Wenbin Meng, **Hongkai Shang,** Shaofu Li, Deborah M Sloboda, Loreen Ehrlich, Karolin Lange, Huaisheng Xu, Wolfgang Henrich, Joachim W Dudenhausen, Andreas Plagemann, John P Newnham, John RG Challis. Reprod Sci. 2014 (ahead of print)

Paper: “Gum Chewing Slightly Enhances Early Recovery from Postoperative Ileus after Cesarean Section: Results of a Prospective, Randomized, Controlled Trial.” **Hongkai Shang**, Yang Yang, Xiaowen Tong, Lijun Zhang, Aiming Fang, Ling Hong. American Journal of Perinatology. 2010; 27(5):387-91.

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Unterschrift des Doktoranden

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Unterschrift, Datum und Stempel des betreuenden Hochschullehrers

11. Affidavit

"I, Hongkai Shang, certify under penalty of perjury by my own signature that I have submitted the thesis on the topic [Effects of maternal dexamethasone treatment early in pregnancy on glucocorticoid receptors in the ovine placenta] I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My interest in any publications to this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date Signature

Declaration of any eventual publications

Hongkai Shang had the following share in the following publications:

Posters:

7th World Congress on Developmental Origins of Health and Disease 2011
"Long term effects of early Dexamethasone treatment on glucocorticoid receptor alpha and the regulation of binucleate cell function in sheep placenta." Hongkai Shang, Wenbin Meng, Deborah M. Sloboda, Shaofu Li, Andreas Plagemann, Joachim W Dudenhausen, John P Newnham, John RG Challis, Thorsten Braun. Developmental Origins of Health and Disease; 2011. p. PI-370.
Contribution in detail: Hongkai Shang performed the lab work, statistical analysis and was involved in writing.

IFPA 2011
"Early Dexamethasone (DEX) exposure has lasting effects on glucocorticoid receptor alpha (GRa) in sheep placentomes." Thorsten Braun, Hongkai Shang, Wenbin Meng, Deborah M Sloboda, Shaofu Li, Andreas Plagemann, Joachim W Dudenhausen, John P

Newnham, John RG Challis; Placenta, Volume 32, Issue 9, A72
Contribution in detail: Hongkai Shang performed the lab work, statistical analysis and was involved in writing.

IFPA 2013
“Sex-specific differences in the sheep placenta.” Thorsten Braun, Wenbin Meng, Hongkai Shang, Shaofu Li, Deborah M Sloboda, Loreen Ehrlich, Huaisheng Xu, Wolfgang Henrich, Joachim W Dudenhausen, Andreas Plagemann, John P Newnham and John RG Challis; Placenta Volume 34, Issue 9, Page A91
Contribution in detail: Hongkai Shang performed the lab work, statistical analysis and was involved in writing.

Manuscripts:
Paper: “Effects of maternal Dexamethasone treatment early in pregnancy on glucocorticoid receptors in the ovine placenta.” Hongkai Shang, Wenbin Meng, Deborah M Sloboda, Shaofu Li, Loreen Ehrlich, Andreas Plagemann, Joachim W Dudenhausen, Wolfgang Henrich, John P Newnham, John R Challis, Thorsten Braun. Reprod Sci. 2015 May; 22(5):534-44.
Contribution in detail: Hongkai Shang performed the lab work, statistical analysis and was involved in writing.

Signature, date and stamp of the supervising University teacher

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Signature of the doctoral candidate

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