Expression of glucocorticoid receptor, mineralocorticoid receptor, and 11β-hydroxysteroid dehydrogenase 1 and 2 in the fetal and postnatal ovine hippocampus: ontogeny and effects of prenatal glucocorticoid exposure

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Abstract

To determine the expression of glucocorticoid metabolizing and action genes in the hippocampus of fetal, neonatal, and adult sheep. Pregnant ewes (or their fetuses) received intramuscular injections of saline or betamethasone (BETA, 0-5 mg/kg) at 104, 111, 118, and/or 125 days of gestation (dG). Hippocampal tissue was collected prior to (75, 84, and 101 dG), during (109 and 116 dG), or after (121, 132, and 146 dG; 6 and 12 postnatal weeks; 3.5 years of age) saline or BETA injections. Hippocampal glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and 11β-hydroxysteroid dehydrogenase (11βHSD)1 and 11βHSD2 mRNA levels were determined using qRT-PCR. Control animals late in gestation demonstrated a decrease in mRNA encoding GR and 11βHSD1, whereas 11βHSD2 was undetectable, consistent with a damping of the negative feedback influence of circulating or locally produced cortisol on the hypothalamic–pituitary–adrenal (HPA) axis. BETA-administration had transient effects on fetal GR and MR, and early in postnatal life (12 weeks of age) 11βHSD1 mRNA was increased. Hippocampal MR mRNA was elevated in adult offspring exposed to either one or four doses of maternal BETA (P<0.001). Four courses of maternal BETA increased 11βHSD2 (P<0.05) but not 11βHSD1 mRNA levels. Late in gestation a reduction in hippocampal GR and 11βHSD1 mRNA suggests lessening of glucocorticoid negative feedback, facilitating increased preterm HPA activity and parturition. Adult offspring of BETA-treated mothers demonstrated increased MR and 11βHSD2 mRNA, therefore it appears that exposure of fetus to high levels of synthetic glucocorticoids may have long-lasting effects on the hippocampal expression of HPA-related genes into adulthood. 


Introduction

A late gestational rise in endogenous plasma cortisol levels occurs in the fetuses of many species and is necessary for tissue and organ maturation as well as for the initiation of parturition (Liggins 1994). This trend corresponds to a widely observed increase in hypothalamic–pituitary–adrenal (HPA) activity in late gestation, but presumably also requires a lessening of negative regulatory control of HPA activity. We have previously reported changes in gene expression in the fetal ovine hypothalamus and pituitary consistent with a late gestational maturation of these organs (Matthews & Challis 1996), but there is limited information on hippocampal gene expression ontogeny in the context of changes in negative regulatory control of HPA activity in late gestation in the fetal sheep (Keller-Wood et al. 2006). Hippocampal corticosteroid receptors (both type 1 (mineralocorticoid receptor, MR) and type 2 (glucocorticoid receptor, GR)) and metabolizing enzymes (11β-hydroxysteroid dehydrogenases, 11βHSD) regulate glucocorticoid action, but their role in fetal HPA axis activation is unclear. It is possible that interaction between these regulators plays a role in the well-known attenuation of negative feedback occurring late in gestation (Matthews & Challis 1996) and facilitates the rise in bioactive cortisol levels necessary for the maturation of fetal organ systems.

While elevated cortisol levels during late pregnancy are required for normal fetal development (Liggins 1994), we have previously shown that inappropriate exposure to endogenous or exogenous glucocorticoids results in a number of adverse outcomes for the fetus, including fetal growth restriction (Newnham et al. 1999), reduced brain growth (Huang et al. 1999), reduced neuronal myelination (Quinlivan et al. 1999), and fetal HPA hyperactivity near term (Sloboda et al. 2000). Studies by
others have shown that early developmental influences affecting HPA axis function may have profound long-term programming changes at the level of the hippocampus (Meany & Aitken 1985, Meany et al. 1989, Levitt et al. 1996).

In the present study, we investigated changes in the expression of four key hippocampal genes in the prenatal sheep, in order to elucidate the contribution of the hippocampus to negative regulatory control of HPA activity during late gestation and early postnatal life. We also examined the effects of exogenous glucocorticoids given to the mother or the fetus on both pre- and post-partum hippocampal GR, MR, and 11βHSD expression levels, in order to determine whether short- or long-term changes in hippocampal gene expression result from exposure to inappropriate levels of glucocorticoids during pregnancy.

Materials and Methods

Experimental procedures were approved by the Animal Experimentation Ethics Committee of the University of Western Australia and/or the Western Australian Department of Agriculture.

Prenatal and postnatal procedures

Prenatal interventions, pregnancy outcomes, and postnatal care of animals have been described in detail previously (Sloboda et al. 2000, Moss et al. 2001). All animals (control and treatment groups) were injected intramuscularly with 150 mg medroxyprogesterone acetate (Depo Provera; Upjohn, Rydalmere, Australia) at 100 dG to reduce pregnancy losses due to subsequent glucocorticoid treatment (Nathanielsz et al. 1988, Jobe et al. 1998). This study involves the combination of two cohorts of animals: Cohort 1, a cohort designed to investigate the effects of maternal betamethasone injection on fetal development throughout gestation and early neonatal life; and Cohort 2, a cohort designed to evaluate long-term effects of prenatal maternal or fetal betamethasone injections on adult sheep offspring.

Cohort 1 – fetal and early postnatal development

Pregnant Merino ewes bearing singleton male fetuses of known gestational age were randomized to receive maternal injections of saline or betamethasone. Pregnant animals received intramuscular injections of saline or one (104 dG), two (104, 111 dG), or three (104, 111, and 118 dG) injections of betamethasone (0.5 mg/kg ewe weight; Celestone Chronodose; Schering Plough, Baulkham Hills, Australia). Saline injections were of a comparable volume (5–6 ml). Hippocampal tissue was collected prior to (75, 84, and 101 dG), during (109 and 116 dG), or after (121, 132, and 146 dG, and at 6 and 12 postnatal weeks of age) either saline or betamethasone injections (Table 1). For fetal tissue collection, pregnant ewes were killed with captive bolt and fetuses were delivered by Caesarean section, weighed, and killed by decapitation. For collection of tissue from neonatal lambs, pregnant ewes were permitted to deliver spontaneously and lambs were kept with ewes until the time of killing. For tissue collection lambs were sedated with ketamine (15 mg/kg) and xylazine (0.1 mg/kg, Troy Laboratories, Smithfield, NSW, Australia) and then killed by decapitation.

Cohort 2 – postnatal adult offspring

Pregnant Merino ewes bearing singleton fetuses were allocated randomly to receive either maternal or fetal intramuscular injections of saline and/or betamethasone (0.5 mg/kg). Saline-treated groups were injected with normal saline at 104, 111, 118, and 125 dG. Single betamethasone dose groups were injected at 104 days of gestation and saline at 111, 118, and 125 days of gestation. Repeated betamethasone dose groups were injected with betamethasone at 104, 111, 118, and 125 days of gestation. Fetal intramuscular injections of betamethasone were given under ultrasound guidance using an established technique (Newnham et al. 1999). We have previously published the results of in vivo HPA experimentation on these offspring at 6 months and 1 year of age (Sloboda et al. 2002), and at 2 and 3 years of age (Sloboda et al. 2007), as well as metabolic experimentation at 6 months and 1 year (Moss et al. 2001) and at 2 and 3 years old (Sloboda et al. 2005). At ~3–5 years of age all offspring were weighed and killed by pentobarbitone overdose (30 mg/kg, Valabarb, Jurox Pty Ltd, Silverwater, Australia).

Offspring were grouped according to prenatal treatments as follows: maternal saline, MS (n=5, male n=3, female n=2) or fetal saline, FS (n=4, male n=1, female n=3); single maternal betamethasone, M1 (n=5, male n=2, female n=3) or single fetal betamethasone, F1 (n=7, male n=4, female n=3); four maternal betamethasone injections, M4 (n=5, male n=2, female n=3); four fetal betamethasone injections, F4 (n=4, male n=2; female n=2).

All hippocampal tissue samples were recovered immediately, snap frozen in liquid N2, and stored at −80 °C until required for RNA extraction and gene expression analyses.

Molecular analyses

RNA extraction and reverse transcription (RT)

Total RNA was extracted using the RNeasy Midi kit...
Quantitative real-time PCR assays  For the quantification of hippocampal MR, GR, and 11βHSD1 and 2, and of the endogenous reference 18S ribosomal RNA (18S), a quantitative PCR assay was performed (Rotor-Gene 3000, Corbett Research, Sydney, Australia). For quantitative PCR all primers were either designed using Primer 3.0 (PE Biosystems; Corbett Research, Sydney, Australia) or have been previously used on ovine tissue (GR, MR, 11βHSD2, 18S; Dodic et al. 2002, Sloboda et al. 2007). Primer sequences for hippocampal MR, GR, 11 βHSD1 and 2 and 18S sets are presented in Table 2. PCRs were carried out in 25 μl volumes containing 10X IMMUNO Buffer (Bioline, Randolph, MA, USA), 3 mM MgCl₂ (Bioline), 10 mM dNTPs (Roche), 0.5 μM forward primer, 0.5 μM reverse primer, SYBR Green (1: 2000; Fisher Biotech, Perth, Australia), and 5 U/μl IMMOLASE DNA polymerase (Bioline).

For tissues collected from fetal and neonatal lambs (Cohort 1), complementary DNA was amplified using the following cycling conditions: 95 °C for 0 s, 62 °C for 15 s, and 72 °C for 10 s. Annealing temperatures varied according to the gene of interest: 62 °C for 18S; 59 °C for MR and GR; and 60 °C for 11βHSD1. In fetal hippocampal samples amplification of 11βHSD2 product was only achievable after >50 cycles. It was therefore deemed that 11βHSD2 mRNA in these samples was beyond the level of detection. The threshold cycle number of detection for each sample was compared against a standard curve constructed using tenfold serial dilutions of corresponding standards specific for each transcript. GR, MR, 11βHSD1, and 18S standards were constructed using serial dilutions of conventional PCR product as a template. All samples for each gene of interest were run in duplicate. Results were expressed as a ratio of transcript (gene of interest) to internal control (18S). Inter-assay coefficient of variation was <10%.

For tissues collected from adult sheep (Cohort 2), complementary DNA was amplified for all transcripts using the following cycling conditions: 95 °C for 0 s, 59 °C for 15 s, and 72 °C for 10 s, for 35 cycles. A comparative cycle of threshold fluorescence (CT) method was used to assess relative gene expression levels, where the CT value reflects the cycle number at which DNA amplification is first detected. Equal amplification efficiencies were confirmed between the gene of interest and the internal control 18S. One control sample within each assay for each gene was used as a calibrator. Thus comparative CT calculations for the expression of hippocampal GR, MR, 11βHSD1, and 2 were all relative to a calibrator. First, 18S CT values were subtracted from hippocampal GR, MR, 11βHSD1, and 2 mRNA values for each sample to give a ΔCT value. ΔΔCT values were achieved by subtracting the calibrator ΔCT value from each sample ΔCT value. The expression of hippocampal GR, MR, 11βHSD1, and 2 relative to the calibrator was evaluated using the expression 2^{−ΔΔCT}. This method of analysis has been previously published using sheep RNA (Dodic et al. 2002, Sloboda et al. 2007). All samples for each gene of interest were run in duplicate in a single assay. Intra-assay coefficient of variation was <5%.

Melting curve analysis demonstrated a single PCR product for MR, GR, 11βHSD1, 11βHSD2, and 18S in all the collected tissues, and the presence of a single band at the appropriate molecular weight was confirmed by gel electrophoresis (data not shown).

Statistical analysis  Data analysis was carried out using Sigma Stat statistical software. To determine changes in mRNA levels due to gestational and
early postnatal age, data from controls in Cohort 1 (75 dG to 12 weeks) were compared using one-factor analysis of variance, with age as the factor. To investigate the effect of betamethasone treatment in Cohort 1, data from tissues collected at 109, 116, 121, 132, and 146 dG and at 6 and 12 weeks of age were compared using two-factor analysis of variance with gestational age and treatment as factors. For Cohort 2, mRNA data were compared using one-factor analysis of variance. In all cases, data that were not normally distributed were log transformed (base e) to achieve data normality. Post-ANOVA comparisons among means were made using the Holm–Sidak method. A \( P \) value of \(<0.05\) was considered statistically significant and all data are presented as mean ± S.E.M.

**Results**

**Hippocampal gene expression during gestation and the early postnatal period in control animals**

Total brain and hippocampal weights increased with advancing gestation (Fig. 1A and B). Levels of MR mRNA in saline controls were relatively constant throughout gestation and any apparent differences were not statistically significant (Fig. 2A). Levels of GR, however, increased between 75 and 109 dG, decreased dramatically between 116 and 121 dG, remained low for the rest of gestation and the immediate post-partum period but rose again at 12 weeks of postnatal age (Fig. 2B). We found that 11βHSD1 mRNA followed a profile similar to that of GR (Fig. 2C); rising between 75 and 109 dG, and then declining to lower values between 116 and 132 dG. 11βHSD2 mRNA was undetectable with the methods used in this study.

**Effects of maternal betamethasone injections on hippocampal gene expression during pregnancy and early postnatal life**

The fetuses of mothers treated with betamethasone had significantly reduced brain weight at 109, 116, and 121 dG as well as at 6 weeks of age (Fig. 1A). Differences in hippocampal weights were not statistically significant (Fig. 1B). Fetal or maternal betamethasone injections (single or repeated) significantly reduced brain weight at 3–5 years of age (Moss et al. 2005). The hippocampal samples were not weighed at postmortem in adult offspring.

Betamethasone had transient effects on the levels of MR and GR mRNA in the fetal hippocampus (Fig. 2A and B); MR was decreased at 109 dG but increased at 116 dG and GR was increased at 116 dG. The ratio of MR to GR was not significantly different between control and betamethasone groups either in the fetuses or in lambs at 6 and 12 weeks of postnatal age (data not shown). The levels of 11βHSD1 mRNA in the fetuses of mothers treated with betamethasone followed a profile similar to the fetuses of control ewes, with the exception that 11βHSD1 mRNA levels at 12-week postnatal age were elevated in exposed fetuses compared with controls (Fig. 2C). 11βHSD2 remained undetectable in betamethasone-exposed fetuses.

**Effects of maternal betamethasone injections on hippocampal gene expression in adulthood**

The levels of MR mRNA in the hippocampus of adult offspring were significantly elevated in animals whose mothers had received one or four injections of betamethasone during pregnancy (Fig. 3A). GR mRNA levels were unchanged (Fig. 3B) and hence MR to GR ratios were significantly increased after in utero betamethasone exposure (MS: 0.99 ± 0.01; M1: 1.92 ± 0.21; M4: 1.67 ± 0.08; \( P < 0.05 \)). The levels of 11βHSD1 mRNA in betamethasone-exposed offspring were not different from controls (Fig. 3C). The levels of 11βHSD2, however, were
significantly increased in those animals whose mothers had received four weekly injections (Fig. 3D, M4). A single maternal injection of betamethasone resulted in an intermediate level of 11βHSD2 mRNA in the offspring of these pregnancies (Fig. 3D, M1). These trends were discernable in both male and female animals (Table 3). The effects of direct fetal injections were variable; there was a decrease in MR mRNA after a single fetal injection at d104 (*P < 0.05) and a decrease in 11βHSD1 after one and four fetal betamethasone injections (*P < 0.05), but no other effects were observed (data not shown).

Discussion

Hippocampal gene expression ontogeny and fetal HPA development in control animals

Little is known about the relationship between hippocampal gene expression ontogeny and control of fetal HPA activity during gestation, but changes in hippocampal function are likely to be implicated in the late-gestational increases in fetal HPA activity, which are observed across many species, and could potentially mediate short- or long-term alterations of HPA function which result from physiological perturbations that the fetus is subjected to in utero.

In this study, we have presented novel data implicating the glucocorticoid metabolizing enzyme 11βHSD1 in the hippocampal regulation of HPA axis function in the ovine fetus. Control fetuses late in gestation demonstrated a dramatic decrease in the levels of mRNA encoding GR and 11βHSD1, suggestive of a potential role for these genes in
lessening the negative feedback influence of circulating or locally produced cortisol on the hippocampal regulation of HPA activity. Our data, together with previous reports (Andrews & Matthews 2000, Keller-Wood et al. 2006), suggest that a reduction in late gestational hippocampal GR could facilitate a rise in fetal adrenocorticotropin (ACTH) and cortisol levels through a release of the hippocampal constraint on HPA activity. Here we further suggest that fetal hippocampal 11βHSD1 may regulate glucocorticoid accessibility to hippocampal GR (and MR) during fetal life by converting local inactive cortisone to bio-active cortisol. Moreover, since peak levels of hippocampal GR and 11βHSD1 occur during the period of maximum brain growth in the ovine fetus (Dobbing & Sands 1979), it could be that both GR, and 11βHSD1 play a role in glucocorticoid-related neuronal development and differentiation. The fact that we were unable to detect mRNA levels of 11βHSD2 suggests that the clearance of endogenous glucocorticoids in the fetal hippocampus by 11βHSD2 may not be a mechanism that contributes to the regulation of the HPA axis in fetal sheep. This observation is at odds with published data on fetal 11βHSD2 development in the fetal rat (Diaz et al. 1998) and strengthens the argument that there are clear species differences in HPA maturation.

Most published data suggest a role for the hippocampus in the negative regulation of HPA axis activity, although there is some evidence that this regulation may be more complex than previously suggested (Tuves et al. 2003). Nevertheless, our data suggest that in ovine fetus, hippocampal GR, and 11βHSD1 may act in concert with hypothalamic and pituitary maturation late in gestation to facilitate both the well-established decrease in the negative feedback regulation of HPA axis activity, and a rise in fetal ACTH and cortisol levels.

Effects of maternal betamethasone injections on hippocampal gene expression during pregnancy and early postnatal life

Since fetus may experience prolonged exposure to both endogenous and exogenous glucocorticoids in utero, and given our previous observations that exposure to glucocorticoids increases fetal HPA axis activity at the term (Sloboda et al. 2000), we aimed to determine whether this would alter

the expression of regulatory genes in the fetal hippocampus. We demonstrated transient changes in hippocampal MR and GR mRNA at 109 and 116 dG after betamethasone exposure. Further work is, however, required to establish whether these observations reflect underlying trends of sufficient magnitude to drive outcomes such as enhanced activity of the fetal HPA axis in the term. We have previously shown that fetal betamethasone exposure did not alter hypothalamic corticotropin-releasing hormone, arginine vasopressin, GR, or pituitary proopiomelanocortin mRNA levels (Sloboda et al. 2000). Based on the data that we report here, if changes in hippocampal gene expression are not responsible for betamethasone-induced changes in HPA activity (Sloboda et al. 2000), it is possible that changes could be driven by alterations at lower levels of the axis such as the adrenal gland.

The increase in 11βHSD1 mRNA levels in 12-week-old offspring is not sustained to adulthood but suggests the potential for locally synthesized glucocorticoids in the hippocampus to influence HPA function.

Effects of maternal betamethasone injections on hippocampal gene expression in adulthood

In contrast to our observations in the fetus, we have demonstrated a significant upregulation of hippocampal MR mRNA in adult offspring of mothers injected with betamethasone. The fact that this increase was observed in adulthood but not in fetal or early postnatal life suggests that this effect may be an indirect consequence of maternal betamethasone administration, rather than a direct effect. We have shown previously that basal ACTH levels are elevated in the face of reduced basal cortisol in these same offspring (Sloboda et al. 2007). Our observed increases in hippocampal MR would be expected to attenuate circulating levels of ACTH and cortisol. We therefore suggest that the upregulation of hippocampal MR mRNA in the current study may be a consequence of reduced levels of circulating cortisol levels (Sloboda et al. 2007) in these same offspring. This is possible since autologous regulation of MR gene expression has been demonstrated previously in the hippocampus of rats (Holmes et al. 1995, Kalman & Spencer 2002). Our observations are most consistent with those reported in the guinea pig (Dean et al. 2001, McCabe et al. 2001, Banjanin

<table>
<thead>
<tr>
<th>Maternal treatments</th>
<th>MS (n = 3)</th>
<th>M1 (n = 2)</th>
<th>M4 (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>0.78 ± 0.14</td>
<td>0.93 ± 0.03</td>
<td>0.73 ± 0.04</td>
</tr>
<tr>
<td>MR</td>
<td>0.97 ± 0.03</td>
<td>1.02 ± 0.03</td>
<td>1.18 ± 0.07</td>
</tr>
<tr>
<td>11βHSD1</td>
<td>0.9 ± 0.05</td>
<td>0.85 ± 0.05</td>
<td>0.94 ± 0.05</td>
</tr>
<tr>
<td>11βHSD2</td>
<td>1.11 ± 0.25</td>
<td>0.87 ± 0.03</td>
<td>1.62 ± 0.41</td>
</tr>
</tbody>
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MS, maternal saline; M1, maternal one dose; M4, maternal four dose groups.

Table 3. Mean relative mRNA levels of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), 11β-hydroxysteroid dehydrogenase (11βHSD)1, and 11βHSD2 in adult sheep (Cohort 2) grouped according to sex. Data are presented as mean ± S.E.M.
et al. 2004), where prenatal dexamethasone administration resulted in reduced basal and stimulated HPA axis function in male offspring and was associated with elevated hippocampal MR (but not GR) mRNA in the dentate gyrus (DG; Liu et al. 2001).

Significant changes in postnatal hippocampal MR/GR balance consistent with our observations reported here may compromise homeostasis and lead to further HPA dysregulation (De Kloet & Derijk 2004). An imbalance in the ratio of MR to GR appears to be influenced by the availability of corticosteroids and co-regulators, and by access to receptors (De Kloet & Derijk 2004), which in the present study could be regulated by changes in the 11βHSD enzymes. Although we did not measure enzymatic activity, it is possible that our observed increase in hippocampal 11βHSD2 mRNA in adult sheep resulted in an elevation in 11βHSD2 activity and a reduction in the cellular availability of endogenous glucocorticoids. A reduction in intracellular glucocorticoid levels in the hippocampus would have downstream effects on central hippocampal negative feedback, through alterations in the accessibility to corticosteroid receptors. It has been shown previously that 11βHSD1 knockout mice had increased HPA activity, attributable to either increased drive and/or attenuated negative feedback (Harris et al. 2001). It is unknown whether 11βHSD2 overexpression in the hippocampus would have the same effects. It is tempting to speculate that an increase in 11βHSD2 would decrease intracellular levels of cortisol in the hippocampus and contribute to the upregulation of MR; but further studies are required to substantiate this.

In order to investigate the effects of synthetic glucocorticoids in the fetal compartment, we have also examined the effects of administering glucocorticoids to the fetus directly, as although synthetic glucocorticoids themselves are not metabolized in the placenta, we have shown that betamethasone may affect placental function (Braun et al. 2007). We found that a single direct fetal betamethasone injection decreased hippocampal MR, but had no net effect on MR to GR ratios, and that repeated injections had no further effect on MR or GR. These data are therefore consistent with our previous reports that HPA axis function is not altered after fetal betamethasone administration (Sloboda et al. 2002, 2007).

Although this study did not involve an analysis of hippocampal sub-regions, our findings do not contradict other studies that have focused on gene expression in specific sub-regions of the brain (Dean et al. 2001, Romeo et al. 2008). We recognize the existence of sex-specific effects of betamethasone on hippocampal gene expression; although the numbers of adult animals available in the present study did not allow us to assess the effect of sex, our observed effects were discernable in both male and female animals.

In summary our measurements have shown 1) that there are changes in hippocampal GR and 11βHSD1, which are consistent with altered ontogenetic feedback, allowing increased fetal HPA axis activity in the term; 2) that although exogenous glucocorticoids have acute effects on fetal hippocampal MR and GR, these effects are not sustained throughout fetal life; and 3) that the administration of synthetic glucocorticoids to pregnant sheep resulted in significant increases in MR and 11βHSD2 gene expression in adult animals, reflecting the potential for longer term alterations in HPA function. These observations suggest a possible role for locally produced glucocorticoids within the hippocampus in facilitating fetal HPA axis activity and possibly brain development. Furthermore, fetal exposure to synthetic glucocorticoids has effects on the hippocampal expression of corticosteroid receptors and metabolizing enzymes – effects that appear to be modified over the lifespan of the offspring.

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