Genomic analysis of neuroendocrine development of fetal brain-pituitary-adrenal axis in late gestation


1Department of Pharmacodynamics and 2Department of Physiology and Functional Genomics, University of Florida, Gainesville, Florida

Submitted 15 July 2005; accepted in final form 4 December 2005

Keller-Wood, Maureen, Melanie J. Powers, Jason A. Gersting, Nyima Ali, and Charles E. Wood. Genomic analysis of neuroendocrine development of fetal brain-pituitary-adrenal axis in late gestation. Physiol Genomics 24: 218–224, 2006. First published December 13, 2005; doi:10.1152/physiolgenomics.00176.2005.—The present study was performed to identify the changes in genomic expression of critical components of the hypothalamus-pituitary-adrenal (HPA) axis in the second half of gestation in fetal sheep. We isolated mRNA from pituitary, hypothalamus, hippocampus, and brain stem in fetal sheep at 80, 100, 120, 130, and 145 days of gestation and 1 and 7 days after delivery. Each group contained four to five fetuses. Using real-time RT-PCR, we measured mRNA expression levels of glucocorticoid receptor (GR), mineralocorticoid receptor (MR), serum- and glucocorticoid-induced kinase-1 (sgk1), proopiomelanocortin (POMC), CRF, and arginine vasopressin (AVP). Both MR and GR were highly expressed in pituitary and hippocampus; in all tissues GR was more highly expressed than MR. AVP was more highly expressed than CRF in hypothalamus. MR, GR, and sgk1 expression were increased postnatally in brain stem, and sgk1 expression was increased postnatally in hypothalamus. GR expression was reduced in pituitary in term fetuses compared with younger ages. Hypothalamic CRF expression was increased at the end of gestation compared with younger ages, and AVP expression was increased in newborn lambs. Pituitary POMC was increased at 100 days of gestation compared with 80 days; hypothalamic POMC was increased at 120 days. Overall, the results demonstrate the expression of both MR and GR in brain regions important for control of the HPA axis. Increases in expression of GR in pituitary at the end of gestation might contribute to the decreased corticosteroid negative feedback sensitivity at term in this species.

sheep fetus; mineralocorticoid receptor; glucocorticoid receptor; proopiomelanocortin; corticotropin-releasing hormone

DEVELOPMENT OF hypothalamo-pituitary-adrenal function is essential for neonatal survival and for normal timing of parturition. In sheep, the fetal adrenal begins secretion of cortisol before parturition, inducing several important enzymes and proteins important for neonatal survival, notably inducing terminal differentiation and secretion of surfactant in the lung (2). The increase in cortisol secretion also increases the expression of CYP17 in placenta and therefore stimulates the conversion of progesterone to estrogen (17). An increase in the estrogen-to-progesterone ratio increases uterine contractility and initiates labor (17). In the sheep the increase in ACTH and cortisol secretion appears to be caused both by maturation of central pathways controlling secretion of ACTH and by a reduction in feedback effects of cortisol just before term (28, 36).

The purpose of this study was to examine the ontogeny of the expression of genes known to be important components of the hypothalamus-pituitary-adrenal (HPA) axis within the brain and pituitary. We examined the induction of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) within areas involved in corticosteroid feedback such as brain stem, hippocampus, hypothalamus, and pituitary. We also measured the expression of mRNA for serum- and glucocorticoid-induced kinase-1 (sgk1), which is a genomic marker of both MR and GR action in various tissues. In postnatal animals, both MR and GR contribute to feedback effects in areas such as hippocampus, and evidence in adult rats suggests that MR are more important in the control of basal ACTH secretion (6). However, in both rats and mice, MR are expressed later in gestation than GR; in situ hybridization demonstrates expression of MR only in the last 1–2 days before birth (7, 29). However, the development of the rodent brain and HPA axis is relatively immature compared with either sheep or primate models, suggesting that MR may be expressed earlier in these species. Finally, we quantified expression of arginine vasopressin (AVP) and CRF in hypothalamus and proopiomelanocortin (POMC) in hypothalamus and pituitary to characterize the timing of increased expression relative to any changes in MR or GR expression.

METHODS

Tissues were collected from fetuses at 80 (80d, n = 4–5), 96–100 (100d, n = 3–4), 120 (120d, n = 4), 130 (130d, n = 4), and 142–144 (145d, n = 4–5) days of gestation and on the first (1d, n = 4) or seventh (7d, n = 4–5) day after delivery. Each group included one set of twin fetuses. None of the ewes showed any signs of impending labor. For collection of fetal tissues, ewes were killed with 20 ml of Euthasol solution (7.8 g pentobarbital and 1 g phenytoin sodium; Virbac AH, Fort Worth, TX) administered intravenously, the fetus was quickly removed, and the fetal brain and pituitary were removed. Fetal tissues were rapidly frozen in liquid nitrogen and stored at −80°C. The use of animals in this project was approved by the University of Florida Institutional Animal Care and Use Committee.

Plasma samples were not collected from these fetuses, as maternal death would be expected to produce changes in fetal ACTH and cortisol and our objective was to obtain fetal tissues rapidly to prevent RNA degradation. Moreover, chronic catheterization to allow blood sampling might also alter gene expression. To assess fetal capacity to secrete cortisol, adrenal glands were collected and adrenal cortex content was measured. The adrenals were rapidly frozen in liquid nitrogen and stored at −80°C until analysis. The use of animals in this project was approved by the University of Florida Institutional Animal Care and Use Committee.

Plasma samples were not collected from these fetuses, as maternal death would be expected to produce changes in fetal ACTH and cortisol and our objective was to obtain fetal tissues rapidly to prevent RNA degradation. Moreover, chronic catheterization to allow blood sampling might also alter gene expression. To assess fetal capacity to secrete cortisol, adrenal glands were collected and adrenal cortex content was measured. The adrenals were rapidly frozen in liquid nitrogen and stored at −80°C until analysis. The adrenals were homogenized in ethanol-saline [50:50 (vol/vol), 1 ml/100 mg adrenal wt], and 0.1 ml of the homogenate was extracted with 1 ml of ethanol. Extracts were dried, and cortisol concentration in the extract was measured.
determined by radioimmunoassay (Cortisol Coat-A-Count kit, Diagnostic Products, Los Angeles, CA). Adrenals were only collected from one of the newborn lambs; therefore, data from both 1d and 7d lambs were analyzed together. We also collected adrenals from five fetuses of five additional ewes showing signs of impending labor to assess changes in adrenal capacity at the time of birth.

RNA was isolated from tissues with TRIzol reagent (GIBCO-BRL, Grand Island, NY) according to the manufacturer’s directions. Total RNA was treated with DNaSe (Ambion DNA-free; Ambion, Austin, TX; 2 U/10 μg RNA), and RNA concentration was subsequently determined by spectrophotometry.

Relative expression of GR, MR, and sgk1 in pituitary, hypothalamus, hippocampus, and brain stem and expression of POMC in pituitary and hypothalamus were determined by quantitative real-time (qRT)-PCR using 5'FAM Taqman probes and primers and Taqman One-step RT-PCR master mix (Applied Biosystems, Foster City, CA). Relative expression of CRF in hypothalamus was determined by two-step qRT-PCR with a high-capacity cDNA archive kit (Applied Biosystems) with 5'FAM probes and primers (Genomechanix, Gainesville, FL). Expression of AVP in hypothalamus was determined by two-step qRT-PCR with the high-capacity cDNA archive kit and SYBR Green I dye (Applied Biosystems SYBR Green PCR Master Mix). Probes and primers were designed with Primer Express software (Applied Biosystems); sequences and concentrations for probe and primer used in quantification of ovine GR, MR, sgk1, and POMC were reported previously (1, 15). Because MR and GR splice variants have not yet been identified in sheep, MR and GR primers were designed within common regions of the gene. Primers were designed from the ovine sequences in GenBank for AVP-neurophysin II precursor mRNA (product corresponds to bases 7–46; AF045248), and CRF probe and primers were designed based on the ovine CRF mRNA (corresponds to bases 580–662; J00803) (11). Sequences and primer concentrations for ovine CRF and AVP primers are shown in Table 1.

Table 1. Sequences and concentrations of primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Taqman Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine Vasopressin</td>
<td>TTCCAGAAGTGGCCCAAGGG</td>
<td>AGAACACGTCTCTAGCTCGAGTCAGGTC</td>
<td>(SYBR Green)</td>
</tr>
<tr>
<td>(250 nM)</td>
<td>(50 nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corticotropin-Releasing</td>
<td>TCCCATTTGCTGGACTCTCA</td>
<td>GAGCTTGCTGGCTAATACCTGA</td>
<td>TTCCACCTCTCCGAGAAGTCTTTGAAAT</td>
</tr>
<tr>
<td>Factor</td>
<td>(300 nM)</td>
<td>(300 nM)</td>
<td>(250 nM)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Both AVP and CRF were expressed throughout the latter half of gestation (Fig. 4). Expression of AVP was much higher than expression of CRF (more than 80-fold greater at 120d and more than 200-fold greater at 145d; Fig. 2). The expression of both levels in 145d fetuses, fetuses of ewes in labor, and newborn lambs.

**MR, GR, and sgk1.** MR, GR, and sgk1 were all expressed within all tissues studied. MR expression was highest in hippocampus, equivalent to expression of GR at 145d (Fig. 2). MR expression in brain stem, hypothalamus, and pituitary was appreciably less than that of GR (Fig. 2), sgk1 expression was high in hippocampus and brain stem relative to GR expression and lowest in pituitary relative to GR.

In hippocampus, the expression of MR and GR did not significantly change with maturation of the fetus (Fig. 3). In brain stem, both MR and GR were significantly increased postnatally (Fig. 3). In hypothalamus, the expression of both MR and GR tended to increase at the end of gestation; however, this change was not statistically significant. (Fig. 4). In pituitary, GR significantly decreased at 145d (Fig. 5).

sgk1, a gene regulated by GR and MR in many tissues, increased in all three brain areas in late gestation. sgk1 was increased after 120d in hippocampus (Fig. 3) and after 100d in hypothalamus and in brain stem (Figs. 3 and 4). In brain stem a further increase occurred in newborn lambs. sgk1 was relatively constant in pituitary (Fig. 5) except for an increase at 145d.

**Hypothalamic expression of CRF and AVP.** Both AVP and CRF were expressed throughout the latter half of gestation (Fig. 4). Expression of AVP was much higher than expression of CRF (more than 80-fold greater at 120d and more than 200-fold greater at 145d; Fig. 2). The expression of both
that focus on gene expression in specific populations of neurons or pituitary cells. However, this simultaneous analysis suggests several hypotheses regarding coordinate maturation and regulation of the axis for further study.

**GR, MR, and sgk1.** Many previous studies have shown that GR is abundantly expressed in ovine fetal brain and pituitary (26, 30, 38). The present study also demonstrates that there is relatively abundant expression of MR in ovine fetal brain, in contrast to the reports of relatively low or no expression of MR in rodents until very late in fetal life (9, 29). The present results suggest that the low level of expression of MR in the rodent before birth is the result of the relative immaturity of the brain-pituitary-adrenal axis in mouse and rat pups at birth, compared with more mature species (33). We suggest that the pattern of expression of MR in the sheep fetus is more likely to be similar to the expression in the human fetus.

Although these studies do not yet demonstrate a physiological role for MR in fetal brain, the presence of MR suggests that corticosteroids might exert actions even in fetal life. Although epithelial cells expressing MR generally also express 11β-hydroxysteroid dehydrogenase (11β-HSD2), previous studies have shown that 11β-HSD2 expression and the associated oxidase activity are low in the late-gestation fetal brain (7, 16), whereas 11β-HSD1 expression is greater than 11β-HSD2 in fetal brain (31). A predominance of 11β-HSD1 oxidoreductase activity could amplify cortisol as a neuroendocrine signal by variably converting cortisone to cortisol, and thereby increasing both MR- and GR-mediated corticosteroid action at the target tissue (10, 32).

The normal ontogenetic pattern of plasma cortisol in sheep has been described by several investigators (27). In fetal sheep, the midgestation adrenal is very sensitive to ACTH and secretes both cortisol and aldosterone (34). After 90 days, fetal adrenal secretion of cortisol and aldosterone decreases. Between 90 and ~130 days, the circulating cortisol in the ovine fetus is primarily derived from transplacental transfer of maternal cortisol (12), resulting in very low circulating fetal cortisol concentrations. Fetal adrenal maturation begins by 130 days and proceeds to term, resulting in an exponential increase in circulating fetal cortisol concentrations peaking at birth. In the fetuses in this study, we also found little adrenal cortisol content at 100 and 120 days, with adrenal cortisol content at the limit of detection of the assay in all four 100d adrenals and in two of four 120d adrenals. Adrenal content was higher in adrenals from 130d fetuses and further increased at 145d, just before birth, and in neonates. The expression of MR in the late-gestation fetal brain suggests the possibility that even the low plasma cortisol levels present before fetal adrenal maturation may exert effects in the brain. In sheep, the estimated $K_d$ for MR is ~0.5 nM, and saturation of MR occurs with cortisol concentrations of ~2–5 nM; the $K_d$ for GR is ~1.5 nM, and saturation occurs at 20–25 nM (24). This suggests that even the low levels of free cortisol (estimated as 1–3 nM in fetuses at 120–130 days of gestation; Refs. 13, 35) present in normal fetuses can act via GR. In adult rats, similar levels of corticosterone (~1.5 nM free) appear to act via MR in the regulation of basal morning ACTH concentrations (6). Our previous observation (14) that reduction of maternal cortisol concentrations by maternal adrenalectomy results in increased fetal ACTH concentrations in 120- to 130-day fetuses suggests that even the normal low concentrations of cortisol in the fetal circulation may exert feedback effects via MR.
The ontogenetic pattern of sgk1 expression differed among the regions studied. sgk1, a marker of corticosteroid action, can be induced in neurons by exposure to corticosteroids, as well as pathologically by ischemia or changes in osmolality (4, 5, 21). Because the brains were all collected from normal fetuses in whom no signs of ischemia or pathology were noted, we expect that sgk1 induction reflects normal ontogenetic physiological changes secondary to brain maturation and changes of the

Fig. 3. Ontogenetic changes in expression of MR, GR, and sgk1 in hippocampus (left) and brain stem (right). Expression of each gene was normalized to β-actin expression in the same sample. Data are mean ± SE fold differences relative to mean expression at 80d. aDifferent from 80d values; bdifferent from 100d values; cdifferent from 120d values; ddifferent from 130d values; edifferent from 145d values; gdifferent from 7th postnatal day (+7d) values. For all statistical comparisons, *P < 0.05 was used as the criterion for significance.

Fig. 4. Ontogenetic changes in expression of MR, GR, and sgk1 (left) and AVP, CRF, and proopiomelanocortin (POMC) (right) in hypothalamus. Expression of each gene was normalized to β-actin expression in the same sample. Data are mean ± SE fold differences relative to mean expression at 80d. aDifferent from 80d values; bdifferent from 100d values; cdifferent from 110d values; ddifferent from 120d values; edifferent from 130d values. For all statistical comparisons, *P < 0.05 was used as the criterion for significance.
hormonal milieu rather than any pathological induction of sgk1. The expression of sgk1 can be induced by either MR or GR activation in various tissues (20, 21). The ontogenetic pattern of sgk1 expression may therefore reveal the responsiveness of these brain areas to steroid action with maturation.

In brain stem, sgk1 is expressed at similar levels in pre- and postnatal lambs, except for an induction in newborn lambs. This suggests that the expected increase in cortisol secretion at the time of birth stimulates sgk1 expression in brain stem. This increase occurs coincident with an increase in both MR and GR expression, suggesting that maturation of brain stem responsiveness to cortisol may occur just before birth. Consistent with this hypothesis, we found strong correlations between sgk1 expression and both MR and GR expression in brain stem (r = 0.805 and r = 0.845, respectively; P < 0.001). In contrast, we found an increase in the abundance of sgk1 mRNA in hippocampus at 130 days. This increase was sustained through birth. As there was no increase in expression of MR or GR in hippocampus at this time, we interpret this result to indicate that MR and/or GR expression by 130 days is adequate to induce sgk1 and that increased sgk1 occurs as a consequence of the increased fetal plasma cortisol concentrations expected to occur at this age. sgk1 expression in hypothalamus and pituitary did not significantly increase until 145d. In fetal hypothalamus, as in adult hypothalami, MR expression is very low (~25% of GR at 145d). Thus sgk1 in hypothalamus may be activated only by the higher levels of cortisol that occur closer to term, mediated primarily by GR. sgk1 expression in hypothalamus also significantly correlated with MR and GR expression (r = 0.694 and r = 0.648, respectively; P < 0.001). The increase in sgk1 expression in pituitary at 145d occurs coincident with a decrease in GR; however, the stimulation of sgk1 by steroids in nonneural and nonnephelithelial tissues has not been investigated. Interestingly, it has been suggested that sgk1 is stimulated in endocrine cells by cAMP (25), so that increased CRF production at term may potentially drive the increase in pituitary sgk1.

**POMC and CRFs.** The pattern of POMC expression in pituitary appears to follow the inverse pattern of adrenal steroid capacity, initially increasing at 100d after fetal steroid production falls, then decreasing from 120d to 130d as steroid production begins to increase. The exception to this pattern is at 145d, when corticosteroid production is high yet POMC expression is increased. The pattern suggests that steroid feedback may regulate POMC expression at all ages except 145d, the time at which pituitary GR were found to be decreased. Thus there is no overall correlation of pituitary POMC with GR expression (r = 0.12). The decrease in GR expression with an increase in POMC expression at term is consistent with physiological data showing that there is reduced sensitivity to cortisol negative feedback inhibition of ACTH at term in fetal sheep (36). Myers and colleagues (3) demonstrated an increase in POMC gene expression in late gestation in corticotropes, reporting a pattern of expression that was similar to the circulating concentrations of cortisol.

Hypothalamic POMC expression increased in late gestation, with the highest levels achieved at 7d postnatally. In adult animals, POMC is expressed in the arcuate nucleus of the hypothalamus and is involved in feeding behavior (8). It is possible that this increase in POMC gene expression in late gestation is an important variable in the establishment of appetite in the newborn lamb.

The patterns of expression of CRF and AVP are consistent with increased activity of the fetal HPA axis at term. Although there was no dramatic increase in expression of CRF at the time of birth, CRF expression increased during the period that corresponds to the time of the normal increase in fetal ACTH and cortisol in this species. This increase in CRF at 130d occurs despite the expected increase in plasma cortisol, suggesting that feedback effects of cortisol at GR must be counterbalanced by other stimulatory inputs to the hypothalamus. The gradual (although not significant) increase in MR, GR, sgk1, and CRF expression in hypothalamus suggests that these may all result from a common maturation process rather than a change in steroid action. Consistent with this hypothesis, we found significant positive relationships between MR and GR expression and both CRF expression (r = 0.864 and r = 0.839,
respectively; \( P < 0.001 \) and AVP expression \( (r = 0.614 \text{ and } r = 0.605, \text{ respectively}; \ P < 0.001) \).

It is interesting that the expression of AVP was greater than that of CRF, reminiscent of previous experiments in other laboratories suggesting the importance of AVP relative to CRF in the control of ACTH secretion in this species (18). Because of the way that we collected the tissue, our hypothalamic AVP mRNA contains expressed mRNA from both parvocellular and magnocellular neurons in both paraventricular and supraoptic nuclei. Indeed, an increased expression of AVP mRNA in late gestation is consistent with increased magnitude of plasma AVP responses to stress (23). Although we do not know the specific cellular localization of the expression pattern for either AVP or CRF in the fetal hypothalamus, the results of these and other experiments (19) are consistent with the results of a study performed by Thorburn and colleagues (22) suggesting that the critical factor in the regulation of adenal cortisol secretion, and therefore of the timing of birth in the sheep, is more related to changes in adrenal cortical sensitivity to ACTH than to a primary change in the synthesis or secretion of the hypothalamic releasing factors controlling ACTH secretion.

We conclude that the genomics of HPA axis development reveal the patterns of endocrine development that drive increasing stress responsiveness and parturition in the ovine fetus. Corticosteroid action, mediated by increased MR and GR expression and highlighted by sgk1 expression, increases in brain stem, hypothalamus, and hippocampus in late gestation. Despite the increase in glucocorticoid stimulation, there is an increase in expression of AVP and CRF in the hypothalamus and an increased POMC gene expression in late-gestation pituitary compared with midgestation. There is evidence of decreased GR in the pituitary, suggesting that this might be the site of reduced cortisol negative feedback sensitivity of the fetal HPA axis at term (36). These results suggest a potential interplay between GR and MR as parallel mediators of corticoid action in the fetal brain and suggest that a normal element of fetal brain development includes increased corticosteroid action in brain stem, hypothalamus, and hippocampus and a lack of increased corticosteroid action at term in pituitary. We conclude that hypothalamus, brain stem, and hippocampus are important sites of corticosteroid action and that the influence of the corticosteroids is likely to be an important component of neuronal development in these brain regions before birth.

ACKNOWLEDGMENTS

We thank Xiaoyang Fang for technical assistance in this study.

GRANTS

This study was supported by National Institutes of Health grants to M. Keller-Wood (DK-62080) for the study of the ontogeny of MR, GR, and sgk1 and to C. E. Wood (HD-42135) for the study of the ontogeny of POMC, CRF, and AVP. J. A. Gersting and M. J. Powers were both supported by Predoctoral Fellowship awards from the Florida-Puerto Rico Affiliate of the American Heart Association. N. Ali was supported by the University of Florida University Scholars Program.

REFERENCES


