Serotonergic effects on feeding, but not hypothalamus-pituitary-adrenal secretion, are altered in ovine pregnancy

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Lingis M, Richards E, Perrone D, Keller-Wood M. Serotonergic effects on feeding, but not hypothalamus-pituitary-adrenal secretion, are altered in ovine pregnancy. Am J Physiol Endocrinol Metab 302: E1231–E1238, 2012. First published February 28, 2012; doi:10.1152/ajpendo.00582.2011.—In ovine pregnancy, as in human pregnancy, hypothalamic-pituitary-adrenal activity is chronically increased. These studies were designed to test the hypotheses that expression of serotonergic genes and responsiveness to serotonin are increased in pregnancy. We tested the stimulatory effect of an acute, intracerebroventricular injection of the serotonin reuptake inhibitor fluoxetine on plasma ACTH and cortisol in ewes during late pregnancy or postpartum. We also tested the effect of lower-dose, longer-term stimulation by intracerebroventricular infusion of fluoxetine in pregnant and nonpregnant ewes over 6 days. Overall, we found that the stimulatory effect of fluoxetine on ACTH and cortisol was not significantly different between late-gestation and nonpregnant ewes, although the effect of acute fluoxetine administration was inversely related to plasma progesterone concentrations. Also, there were no differences in hypothalamic expression of the glucocorticoid and mineralocorticoid receptors, corticotropin-releasing hormone, AVP, the serotonin reuptake transporter, or the serotonin [5-hydroxytryptamine (5-HT)] receptors 5-HT1A and 5-HT2A with pregnancy or fluoxetine treatment. However, chronic fluoxetine infusion reduced food intake in the nonpregnant, but not pregnant, ewes. Expression of proopiomelanocortin mRNA in the hypothalamus was reduced in pregnant compared with nonpregnant ewes. Our results indicate that pregnancy does not increase responsiveness of ACTH and cortisol to serotonergic stimulation but, rather, that progesterone reduces the ACTH response. In addition, we found a reduced ability of serotonin to inhibit feeding in the pregnant ewes, consistent with a reduction in anorexie mechanisms in the pregnant state.

adrenocorticotropic hormone; cortisol; proopiomelanocortin; serotonin

THE SEROTONERGIC SYSTEM is one of the stimulatory inputs to the hypothalamic-pituitary-adrenal (HPA) axis (9, 15). In the rat, serotonergic axons synapse on neurons in the paraventricular nucleus of the hypothalamus (PVN), which contain corticotropin-releasing hormone (CRH), and serotonin directly stimulates CRH release in hypothalamic cultures (10). In vivo administration of serotonin [5-hydroxytryptamine (5-HT)], its precursors, or 5-HT receptor agonists in humans and animal models, including sheep, increases plasma ACTH and cortisol (7, 9, 15, 21–23, 26; reviewed in Ref. 12). Selective serotonin reuptake inhibitors (SSRIs), which act on the transporter responsible for removing serotonin from the synaptic cleft, also acutely increase plasma ACTH concentrations (16, 22). Serotonin-induced stimulation of ACTH involves hypothalamic 5-HT1A and 5-HT2A receptors (reviewed in Ref. 12).

Studies in several species suggest that gonadal steroids may alter responsiveness of the HPA axis to serotonergic stimulation. Acute responses to a SSRI are greater in intact and ovariectomized female than male rats and are reduced by treatment with testosterone (17). Estrogen had no consistent effect on the acute ACTH response to a 5-HT1A receptor agonist (14) but produced a desensitization of 5-HT1A receptors in the hypothalamus (37). Although serotonergic stimulatory drugs such as SSRIs are used with great caution in pregnancy because of effects on fetal cardiac development (35), little is known about the relative effects of SSRIs on maternal HPA function in pregnancy. In studies of women (11) and ewes (1), basal plasma ACTH and cortisol have been shown to increase during pregnancy. The goal of this study was to determine if there are alterations in the serotonergic activation of basal HPA axis stimulation during late pregnancy. We have used the SSRI fluoxetine to exploit the inherent serotonergic activity in pregnant and nonpregnant ewes.

MATERIALS AND METHODS

Animals

Animals were housed in climate-controlled, individual pens in the University of Florida Animal Care Facility; all animal use conformed to the rules and regulations of the Institutional Animal Care and Use Committee at the University of Florida, which approved the protocol. All ewes were given ad libitum access to food (Teklad Ruminant lab diet, Harlan Laboratories, Tampa, FL) throughout the study period; the food was weighed each morning, and food intake was calculated for the previous day.

Study I. We tested the HPA responses to acute, intracerebroventricular injection of fluoxetine, a SSRI, in pregnant (n = 6; 134–137 days of gestation; full term ~147 days) ewes of mixed Western breeds. Animals were allowed to deliver, the lambs were removed, and the ewes were studied again in the postpartum state (8 ± 3 days postpartum). In previous studies, we found that maternal ACTH and cortisol concentrations, as well as feedback responses to cortisol, return to nonpregnant levels as soon as 5 days postpartum (25).

Study II. We tested HPA responses to continuous, intracerebroventricular infusion of fluoxetine in pregnant (117–126 days of gestation) and nonpregnant mixed Western breed ewes. Prior to surgery, nonpregnant and pregnant animals were randomly assigned to receive fluoxetine (n = 6 nonpregnant and 7 pregnant) or vehicle (n = 5 each nonpregnant and pregnant). The duration of infusion (6 days) is shorter than the duration that produces changes in mineralocorticoid receptor (MR), glucocorticoid receptor (GR), or CRH expression with more chronic dosing (2–4 wk), as in therapeutic treatment for clinical depression (4, 28, 34).

Surgical Protocol

Before surgery, food was withheld from the ewe for 24 h. All surgeries were performed in the surgery suite of the Health Science Center Animal Resources Department. Animals were prepared for and underwent surgery under aseptic conditions. Anesthesia was induced

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with isoflurane and maintained using isoflurane or halothane inhalant (1–3% in oxygen).

For infusion of drug or vehicle, a sterile polyvinyl catheter (0.030 in. -ID at the intracerebroventricular tip affixed to a 0.040-in.-ID catheter prior to sterilization) was placed into the lateral ventricle of the ewe. Briefly, a sterilized portable rotary drill (Dremel, Robert Bosch Tool, Racine, WI) was used to make a small hole in the skull, ~3 mm to the right of bregma, and a 21-gauge needle was lowered through the hole until cerebrospinal fluid flowed into the hub. This depth was marked, and a catheter was advanced through the hole to the same depth. The hole in the skull around the catheter was filled with bone wax (CP Medical, Portland, OR), and Vetbond (3M, St. Paul, MN) was used to secure the catheter to the surrounding skull. The position of the catheter tip was verified at necropsy.

In animals prepared for study I, the free end of the intracerebroventricular catheter was externalized, sutured in place, and plugged using a sterile 16-gauge brass nail. The external portion of the catheter was protected under Vetwrap (3M) until intracerebroventricular access was needed on the day of the experiment. A jugular catheter was also placed in these ewes at the time of surgery for subsequent collection of blood samples.

In animals prepared for study II, the free end of the intracerebroventricular catheter was affixed to an Alzet osmotic pump (5 μL/h; model 2ML2, Durect, Cupertino, CA), which was filled at the time of surgery with vehicle (50% DMSO in saline or H2O) or fluoxetine (5 mg/ml; estimated daily dose of 0.6 mg/day or 99 μg.kg⁻¹.day⁻¹ into the lateral ventricle) and positioned in a subcutaneous pocket created near the base of the skull. This dose was expected to produce levels within the brain similar to therapeutic doses of 20–80 mg/day for a similarly sized rabbit. The catheter volume from the ventricles to the osmotic pump (4 cm, 0.30-in.-ID tip and 28 cm, 0.040-in.-ID extension for a volume of 0.24 ml) was designed to deliver vehicle for ~48 h prior to delivery of the contents within the Alzet pump. Sterile polyvinyl catheters were placed in both femoral arteries and veins, as previously described (1).

In studies I and II, during the 5 days immediately following surgery, the animals received twice-daily intramuscular injections of ampicillin (1 g); body temperature was also monitored twice daily.

**Experimental Protocol**

In studies I and II, we used a previously described method to obtain blood samples from the ewes without entering the pen (1); a catheter was externalized from the pens via a swiveling duct system prior to each experiment, and the animals were allowed to acclimate for ~1 h before the start of the experiment.

**Study I.** After 5 days of postoperative recovery, experiments were conducted. Each ewe was injected with fluoxetine (3.3 mg or 77 ± 2 μg/kg in 1 ml of saline over ~20 s) or saline using a crossover design; the two experiments were performed ±2 days apart. For each experiment, a baseline blood sample (8 ml) was collected prior to the injection, and blood samples were collected every 10 min for 60 min postinjection. The blood was collected into tubes containing EDTA (400 μl of 0.3 M EDTA), and the tubes were centrifuged at 3,000 g for 20 min at 4°C; aliquots of plasma were stored at ~20°C for analysis of plasma ACTH and cortisol. After delivery of the lamb, the newborns were removed, and the experiments were repeated in the postpartum state.

**Study II.** On the morning of postoperative day 2 (day 0), a baseline plasma sample was collected as described for study I; an additional 1.5 ml of blood was collected into heparinized syringes and used for determination of sodium and potassium concentrations using ion-specific electrodes (AVL 9180 Electrolyte Analyzer, AVL/Roche Diagnostics, Roswell, GA), as well as for determination of hematocrit and total protein. Mean arterial blood pressure (MAP) was determined using an arterial catheter via an analog-to-digital conversion board (LabView, National Instruments, Austin, TX). After the animals were allowed to acclimatize for 1 h, blood pressure was continuously recorded at 30 Hz for 40–60 min. Two additional blood samples were collected from the arterial catheter: one at the end of the recording period and one ~30 min later. This protocol was repeated every other day through postoperative day 8 (or 6 continuous days of drug infusion, day 6).

**Plasma hormone determination.** All plasma samples were analyzed for ACTH and cortisol concentrations, and the first sample collected each day was analyzed to determine the plasma progesterone concentration. Plasma ACTH concentrations were determined using a RIA previously described using an antibody to ACTH-(1–39) (1). Plasma cortisol and progesterone concentrations were determined by RIA (Coat-A-Count cortisol and progesterone kits, Siemens Healthcare Diagnostics, Deerfield, IL). Coefficient of variation for the cortisol assays for each study was 10% for pools of mean values of 5.4 and 8.6 ng/ml; coefficient of variation for the ACTH assays was 18% for a pool of mean value of 78 pg/ml and 12% for a pool of mean value of 402 pg/ml.

For study I, the effects of pregnancy and acute intracerebroventricular fluoxetine administration on plasma levels of ACTH and cortisol were determined using three-way ANOVA with repeated measures over pregnancy status, drug, and time. For study II, the effects of pregnancy and 6 days of intracerebroventricular fluoxetine administration on blood pressure and plasma levels of cortisol, ACTH, and hematocrit over time were analyzed using a three-way repeated-measures ANOVA of the between-subjects effects of group and treatment and the within-subjects effects of time using SPSS (IBM). Pair-wise multiple comparisons were performed using Fisher’s least significant differences contrasts in SPSS. Values are means ± SE. P < 0.05 was considered significant.

**Gene Expression in the Hypothalamus**

Hypothalami were collected from the ewes in study II on the afternoon of day 6 of fluoxetine or vehicle infusion. Hypothalami were also obtained from a separate cohort of untreated nonpregnant (n = 7), pregnant (130–146 days of gestation, n = 10), and postpartum (4 ± 1 days postpartum, n = 12) ewes. Ewes were euthanized with an intravenous injection of a pentobarbital-phentoyin solution (15–20 ml; Euthasol, Virbey, Fort Worth, TX) via an indwelling venous catheter. Ewes were euthanized in their home pens to minimize stress during this procedure and immediately transported to the necropsy room. The carotid arteries were catheterized, and the brain was perfused with ice-cold 10% DMSO-0.9% saline solution. After dissection of the brain, the whole hypothalamus was immediately snap-frozen in liquid nitrogen and stored at ~80°C for future mRNA quantification.

The hypothalami from the ewes in study II and those from the cohort of untreated nonpregnant, pregnant, and postpartum ewes were analyzed separately. In each group of animals, RNA was extracted by homogenization in TRIzol (GIBCO/BRL, Grand Island, NY) according to the manufacturer’s directions. Genomic DNA was removed using RNeasy Plus Mini Kits with on-column DNase treatment (Qiagen, Valencia, CA). The absorbance of a diluted aliquot of each sample was then measured at 260 and 280 nm for determination of RNA concentration and purity using a spectrophotometer (model ND-1000, Nanodrop Technologies, Wilmington, DE).

Reverse transcription was performed using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA); the cDNA was stored at ~20°C. Quantitative PCR was then performed, and analysis was carried out using the TaqMan Universal PCR Master Mix and the Prism 7000 sequence detection system according to the manufacturer’s instructions (Applied Biosystems). The relative mRNA expression levels in the hypothalamus of the following genes were investigated: MR, GR, CRH, AVP, 5-HT₁A and 5-HT₂A receptors, serotonin transporter (SERT), neuropeptide Y (NPY), and proopiomelanocortin (POMC). The template amount for each gene was 20 ng of cDNA,
Table 1. HPA-related gene expression in hypothalami

<table>
<thead>
<tr>
<th>Gene</th>
<th>Nonpregnant</th>
<th>Pregnant</th>
<th>Postpartum</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>1.07 ± 0.07</td>
<td>1.22 ± 0.21</td>
<td>1.17 ± 0.10</td>
</tr>
<tr>
<td>MR</td>
<td>1.20 ± 0.31</td>
<td>0.75 ± 0.06</td>
<td>0.80 ± 0.07</td>
</tr>
<tr>
<td>CRH</td>
<td>1.35 ± 0.33</td>
<td>1.96 ± 0.56</td>
<td>1.45 ± 0.25</td>
</tr>
<tr>
<td>AVP</td>
<td>1.26 ± 0.37</td>
<td>0.72 ± 0.18</td>
<td>0.64 ± 0.15</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>1.10 ± 0.22</td>
<td>0.99 ± 0.15</td>
<td>0.98 ± 0.12</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>1.04 ± 0.13</td>
<td>1.02 ± 0.11</td>
<td>1.26 ± 0.15</td>
</tr>
<tr>
<td>SERT</td>
<td>1.04 ± 0.13</td>
<td>1.06 ± 0.15</td>
<td>0.98 ± 0.07</td>
</tr>
<tr>
<td>POMC</td>
<td>1.16 ± 0.30</td>
<td>0.34 ± 0.07*</td>
<td>0.47 ± 0.12*</td>
</tr>
</tbody>
</table>

Values (means ± SE) are expressed as fold change relative to nonpregnant ewes. All animals were untreated. HPA, hypothalamic-pituitary-adrenal; GR and MR, glucocorticoid and mineralocorticoid receptors; CRH, corticotropin-releasing hormone; 5-HT1A and 5-HT2A, serotonin [5-hydroxytryptamine (5-HT)] receptors; SERT, serotonin transporter; POMC, proopiomelanocortin. *Significantly different from ΔCT of nonpregnant group (P < 0.05).

except for MR, which was analyzed using 100 ng of cDNA. The probe and primer sequences for MR, GR, CRH, POMC, and AVP are published elsewhere (24). The primers and probes for 5-HT1A and 5-HT2A receptors and SERT and the final concentrations at which they were used were as follows: GCCGACCCGGAAAGA (forward, 900 nM), GTCACCTATGTGTC (reverse, 300 nM), and CGGAGGACTTGGCCAGATAC (probe, 250 nM) for 5-HT1A; TGTGCCTGGTGATGAGATTGATG (reverse, 900 nM), and AGAACCCCATCCATCA (forward, 900 nM), TGACCAGCGCAAGCA (reverse, 900 nM), and AGACGTCTGGGCCAA (probe, 250 nM). All probe and primer sets were validated for specificity against the intended gene using BLAST software and for efficiency using a 100-fold range of primer sets. The NPY primer set was validated for specificity and efficiency using a 100-fold range of primer sets. The NPY primer sequences were as follows: CGGAGGACTTGGCCAGATAC (forward, 900 nM), ACTCAGCGCTGCGAC (probe, 250 nM). All probe and primer sets were validated for specificity against the intended gene using BLAST software and for efficiency using a 100-fold range of input cDNA. All samples for a given gene were run in triplicate on a single 96-well plate. β-Actin was run for each sample as the housekeeping gene and did not change with pregnancy status or fluoxetine treatment.

In the set of untreated ewes, POMC and AVP amplification in two of the nonpregnant ewes was late relative to the rest of this group (shifts of 16- to 128-fold for POMC and 500-fold for AVP compared with other samples) and were excluded from the analyses; therefore, “n” for the nonpregnant group was 5. We hypothesize that imperfect dissection of these two hypothalami may have resulted in partial loss of the arcuate and lateral paraventricular nucleus. For 5-HT2A receptor, fewer samples (n = 4 nonpregnant, 10 pregnant, and 10 postpartum) were available at the time of the analysis.

One-way ANOVA was performed using the relative cycle threshold (ΔCT) method (31). ΔCT was calculated as the mean CT for a set of triplicates in a given sample for β-actin minus the mean CT for a set of triplicates in the same animal for the gene of interest. One-way ANOVA was performed using ΔCT values. Post hoc analysis was performed using Duncan’s multiple range test. On data sets that were not normally distributed and/or had unequal variance, Kruskal-Wallis one-way ANOVA on ranks was performed. The criterion for significance for all statistics was P < 0.05. Relative gene expression or fold change for each gene of interest was calculated as 2−ΔΔCT, where ΔΔCT was calculated as ΔCT for a given animal minus the mean ΔCT for the nonpregnant group.

RESULTS

Changes in Gene Expression During Pregnancy

There were no significant differences in expression of GR, MR, CRH, AVP, SERT, or 5-HT1A or 5-HT2A receptors in hypothalami from a cohort of untreated pregnant, nonpregnant, and postpartum ewes (Table 1). Expression of POMC mRNA was significantly lower in pregnant and postpartum than nonpregnant ewes (P < 0.05; Table 1).

Study I

HPA responses to acute, intracerebroventricular injection of fluoxetine in pregnant and postpartum ewes. Mean plasma ACTH concentrations after injection of vehicle were greater in ewes during pregnancy than postpartum (120 ± 28 vs. 58 ± 8 pg/ml, main effect of pregnancy in 2-way ANOVA within vehicle injection experiments, 1-tailed P < 0.05). In ewes during pregnancy or postpartum, fluoxetine injection stimulated plasma ACTH (main effects of time and treatment, interaction of treatment and time, P < 0.05; Fig. 1). No overall differences between ACTH responses of the ewes during pregnancy and during the postpartum period were revealed in the three-way ANOVA. However, comparison of the responses to fluoxetine by two-way repeated-measures ANOVA and post...
hoc analysis revealed that, at 30 and 40 min postinjection, the plasma ACTH responses were significantly greater postpartum than during pregnancy. Assessment of ACTH responses in each animal revealed that, in four of six ewes, the ACTH responses were reduced during pregnancy compared with postpartum and, in the remaining two ewes, the ACTH responses during pregnancy closely resembled their respective postpartum responses to fluoxetine. Although the mean plasma progesterone concentration of the six ewes during pregnancy was $12.6 \pm 2.6$ vs. $0.2 \pm 0.1$ ng/ml in the postpartum state, the two ewes with the ACTH responses that were similar in pregnancy and postpartum also had lower plasma progesterone concentrations during pregnancy ($4.4$ and $5.8$ ng/ml) than the other 4 ewes ($11.1$–$22.1$ ng/ml). Linear regression analysis revealed a significant inverse relationship between plasma progesterone at the time of fluoxetine injection and the peak ACTH response to intracerebroventricular fluoxetine (Fig. 2; $r = -0.687, P < 0.05$).

Plasma cortisol concentrations were greater in the ewes during pregnancy than postpartum ($15.2 \pm 3.3$ vs. $8.3 \pm 2.5$ ng/ml, main effect of pregnancy by 3-way repeated-measures ANOVA, $P < 0.05$). During pregnancy and postpartum, there was a significant increase in plasma cortisol in response to intracerebroventricular fluoxetine injection (main effects of treatment and time, interaction of treatment and time, $P < 0.05$; Fig. 1). However, cortisol concentrations were at or near maximum adrenal secretion for this species; therefore, neither pregnancy status nor progesterone concentration significantly altered the acute cortisol responses to intracerebroventricular treatment with fluoxetine.

**Study II**

HPA responses to intracerebroventricular infusion of fluoxetine for 6 days in pregnant and nonpregnant ewes. Overall, plasma ACTH and plasma cortisol were not significantly different between pregnant and nonpregnant ewes during vehicle treatment. Three-way repeated-measures ANOVA revealed an effect of fluoxetine treatment over time (interaction of treatment and time, $P < 0.05$). There was also an effect of pregnancy status over time (interaction of pregnancy status and treatment, $P < 0.05$) on ACTH, but no interaction between pregnancy status and treatment effect (Fig. 3). However, the time course of the plasma ACTH responses to intracerebroventricular infusion of fluoxetine tended to differ between nonpregnant and pregnant ewes (interaction of time, pregnancy status, and treatment, $P = 0.057$; Fig. 3), and the increase in cortisol over time was only significant in the fluoxetine-treated nonpregnant ewes ($P < 0.05$ by ANOVA). Post hoc tests revealed that plasma ACTH concentration was transiently increased on day 2 of the fluoxetine infusion in the pregnant ewes compared with the same ewes on day 0 or the vehicle-infused pregnant ewes on day 2, whereas plasma ACTH concentration in nonpregnant ewes remained unchanged compared with vehicle until day 6. Plasma cortisol concentrations followed a similar pattern; although there were no overall interactions of the effects of treatment and pregnancy status over time on plasma cortisol, there were significant increases in plasma cortisol on day 6 in nonpregnant ewes during fluoxetine treatment (Fig. 3, C–F). Interestingly, however, the plasma cortisol concentrations during fluoxetine infusion were highly variable across the three samples taken on each day of the study compared with vehicle infusion in both groups of ewes (Fig. 3, E and F).

**Food intake during intracerebroventricular infusion of fluoxetine.** Given free access to the normal ruminant laboratory diet, nonpregnant and pregnant ewes ate similar amounts of food during the vehicle infusion (Fig. 4); in both groups of ewes, there was a significant increase in food intake over the time of the study, as expected in the postsurgical period. Although there were no main effects of group or treatment on food intake, fluoxetine significantly altered food intake over time (interaction of time and drug, $P < 0.05$). There was also a difference between pregnant and nonpregnant ewes in the response to fluoxetine (interaction of pregnancy status and drug and 3-way interaction of time, pregnancy, and drug, $P < 0.05$). The feeding pattern in nonpregnant ewes during fluoxetine treatment was significantly different from that during vehicle treatment (Fig. 4). In the nonpregnant ewes, food intake was significantly reduced on days 2–3 of fluoxetine infusion compared with previous days in the same animals, whereas food intake was significantly increased over time in vehicle-infused nonpregnant ewes. Food intake was significantly lower on days 4–5 in the nonpregnant ewes treated with fluoxetine than in those infused with vehicle. In the pregnant ewes, however, there was no significant change over time in food intake during fluoxetine infusion.

**MAP and hematocrit during intracerebroventricular infusion of fluoxetine.** Systemic serotonergic drugs can have cardiovascular effects; therefore, to assess potential indirect effects of fluoxetine administration, blood pressure, packed cell volume, and electrolyte concentrations were measured. Three-way repeated-measures ANOVA revealed no significant effects of group or fluoxetine infusion on MAP. Hematocrit was also not altered by treatment with fluoxetine, but in nonpregnant ewes, hematocrit decreased over time (from $32.0 \pm 1.8$ to $28.5 \pm 1.2%$ and from $32.5 \pm 0.8$ to $28.2 \pm 1.4%$ in vehicle-
and fluoxetine-treated nonpregnant animals, respectively, and from 31.2 ± 1.5 to 30.1 ± 1.4% and from 28.2 ± 1.7 to 28.6 ± 1.6% in vehicle- and fluoxetine-treated pregnant animals, respectively.

**Gene expression during intracerebroventricular infusion of fluoxetine.** Hypothalami from ewes in study II revealed no effect of fluoxetine treatment on expression of MR, GR, CRH, AVP, NPY, or POMC, nor did fluoxetine alter 5-HT1A or 5-HT2A receptor expression (Table 2). However, POMC expression was reduced in pregnant compared with nonpregnant ewes, regardless of fluoxetine treatment (Table 2).

**DISCUSSION**

**Effects on ACTH**

These results indicate that responsiveness to serotonergic stimulation is not increased in pregnancy. Our results suggest that although there are no sustained increases in ACTH or cortisol responses to serotonergic stimulation in pregnancy, increased progesterone in pregnancy may tend to reduce ACTH responses to acute fluoxetine stimulation. This effect is not the result of altered hypothalamic expression of the 5-HT1A or 5-HT2A receptors in pregnancy or alterations in regulation of these receptors by fluoxetine.

The results suggest that there may be a blunting of serotonin-stimulated HPA axis activation during acute stress in the presence of progesterone levels typical of pregnancy. The effect of chronic increases in progesterone on the serotonergic system in the hypothalamus is not clear. Several studies suggest that progesterone would increase serotonin release and binding in the nonhuman primate (reviewed in Ref. 2). In ovariectomized ewes, treatment with estradiol and progesterone to normal luteal phase levels for 3–4 wk did not alter the ACTH response to the SSRI sertraline or the 5-HT1A agonist buspirone (6–8); however, the progesterone levels in four of the six ewes in study I and all the ewes in study II exceeded those of the luteal phase, suggesting that only higher levels of progesterone, as in pregnancy, might exert this effect. Estrogen, which is also increased in pregnancy, attenuates 5-HT1A receptor-stimulated increases in ACTH and corticosterone in

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**Fig. 3. Mean plasma ACTH (A and B) and cortisol (C–F) responses to intracerebroventricular infusion of vehicle or fluoxetine in pregnant (filled symbols) and nonpregnant (open symbols) ewes.**

A–D: means of 3 samples collected each day from each ewe. Values are group means ± SE and considered significant at P < 0.05. **Significantly greater than vehicle-infused ewes on the corresponding day of infusion. †Concentration significantly different between pregnant and nonpregnant states. *Significantly different from day 0. E and F: mean group cortisol data from pregnant (filled bars) and nonpregnant (open bars) ewes. Values (means ± SE for each of the 3 daily samples) reflect the greater variability during fluoxetine infusion.
rats through a reduction of G proteins, which are known to mediate the actions of this receptor (32). More recently, the estrogen receptor-β and GPR30 have been implicated in the downregulation of 5-HT1A receptors (33, 37).

Some progesterone actions in the brain are also thought to be mediated by its neurosteroid metabolites, dihydroprogesterone and tetrahydroprogesterone, which activate inhibitory GABAA input, thereby providing a mechanism for progesterone to alter the stimulation of ACTH by fluoxetine or oppose fluoxetine effects in the PVN. It is also known that progesterone metabolism in the brain is altered by fluoxetine, which lowers the Km of rat brain 3α-hydroxysteroid dehydrogenase type 2 for 5α-dihydroprogesterone (18) and would augment effects of the progesterone-derived neurosteroids. Therefore, it is possible that, in the ewe, progesterone, perhaps in conjunction with the estrogenic environment of pregnancy, reduces serotonergic responsivity of the HPA axis. The result of the genomic analysis performed in the ovine hypothalamus suggests that this effect is not mediated by a change in the expression of the genes for SERT or 5-HT1A or 5-HT2A receptors. Further studies are needed to replicate and determine the mechanism of the apparent effect of progesterone.

Studies in rats suggest that the increased corticosteroid levels of ovine pregnancy could also contribute to reduced responses to the SSRI. In rats, changes in circulating corticosteroid levels, produced via exogenous administration or adrenalectomy, result in inverse changes in the abundance of postsynaptic 5-HT1A receptor mRNA and protein in the hippocampus, an upstream modulator of HPA axis activity (5, 12, 13, 27). Chronic increases in corticosterone over 14 days also desensitize 5-HT2A receptors within the PVN (29). However, in our studies, there were no changes in mRNA for 5-HT1A or 5-HT2A receptors during pregnancy, a state of chronically increased corticosteroids, nor was there a change in 5-HT1A receptor binding in the hippocampus of pregnant vs. nonpregnant ewes (unpublished data), suggesting that neither the effect of progesterone nor the increase in plasma cortisol during ovine pregnancy is sufficient to cause changes at the transcriptional level for these serotonin receptors.

**Effects on Feeding**

The differential effect of fluoxetine treatment on food intake between the pregnant and nonpregnant ewes suggests an alteration in central serotonin-mediated satiety pathways during pregnancy. Reductions in meal size and food intake are observed following peripheral or central injections of serotonergic agents, and these changes are consistent with alterations in

![Figure 4](http://ajpendo.physiology.org/)

**Table 2. HPA-related gene expression in hypothalamus following 6 days of intracerebroventricular fluoxetine infusion**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Vehicle Nonpregnant</th>
<th>Fluoxetine Nonpregnant</th>
<th>Vehicle Pregnant</th>
<th>Fluoxetine Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>1.12 ± 0.22</td>
<td>0.90 ± 0.15</td>
<td>0.94 ± 0.05</td>
<td>0.75 ± 0.07</td>
</tr>
<tr>
<td>MR</td>
<td>1.12 ± 0.30</td>
<td>0.66 ± 0.17</td>
<td>2.05 ± 0.07</td>
<td>1.23 ± 0.31</td>
</tr>
<tr>
<td>CRH</td>
<td>1.38 ± 0.62</td>
<td>4.03 ± 1.65</td>
<td>3.90 ± 1.27</td>
<td>4.49 ± 1.35</td>
</tr>
<tr>
<td>AVP</td>
<td>2.21 ± 1.53</td>
<td>2.49 ± 0.82</td>
<td>4.07 ± 0.84</td>
<td>4.02 ± 1.06</td>
</tr>
<tr>
<td>5-HT1A</td>
<td>1.20 ± 0.38</td>
<td>0.76 ± 0.20</td>
<td>0.70 ± 0.13</td>
<td>0.64 ± 0.11</td>
</tr>
<tr>
<td>5-HT2A</td>
<td>1.12 ± 0.21</td>
<td>1.17 ± 0.23</td>
<td>1.39 ± 0.25</td>
<td>0.88 ± 0.16</td>
</tr>
<tr>
<td>POMC</td>
<td>1.31 ± 0.61</td>
<td>2.27 ± 1.36</td>
<td>0.44 ± 0.13*</td>
<td>0.35 ± 0.04*</td>
</tr>
<tr>
<td>NPY</td>
<td>1.41 ± 0.35</td>
<td>1.76 ± 0.41</td>
<td>1.54 ± 0.41</td>
<td>1.20 ± 0.22</td>
</tr>
</tbody>
</table>

Values (means ± SE) are expressed as fold change relative to nonpregnant control ewes. NPY, neuropeptide Y. *Significantly different from ΔCt of nonpregnant control group (P < 0.05).
the mechanisms of satiety (3). In both groups of vehicle-treated ewes, food intake increased over time, as expected in ewes postoperatively. In the nonpregnant, fluoxetine-treated ewes, food intake decreased, rather than increased, over time, consistent with the previously published results (3, 36). This reduction in food intake during fluoxetine infusion did not occur in the pregnant ewes. Serotonin’s role in regulation of food intake is thought to be mediated, at least in part, through its stimulatory effects at the POMC neurons of the arcuate nucleus of the hypothalamus (19, 20). POMC neurons exert an anorectic effect on food intake, and reduced POMC expression in the hypothalamus of the pregnant ewe would therefore increase food intake in pregnancy. The ability of increased serotonergic tone to stimulate melanocortin release may be attenuated in the pregnant ewe. In contrast, there was no effect of pregnancy on NPY mRNA, nor did the SSRI alter expression of this orexogenic peptide in the hypothalamus.

In summary, our results indicate no change in expression of genes for the serotonergic receptors within the hypothalamus during pregnancy and no change in the ACTH response to serotonin reuptake inhibition during pregnancy, therefore suggesting that increased serotonergic tone is not the primary contributor to increased ACTH secretion in the pregnant ewe. Our previous studies suggest that alterations in corticosteroid feedback effects through mineralocorticoid action and increased sensitivity to stresses, such as perceived hypovolemia, are more likely contributors to the increase in plasma ACTH (30). In contrast, our results do indicate that the role of serotonin in control of appetite may be altered in pregnancy. This appears to be a reflection of the reduced POMC expression within the hypothalamus during pregnancy, which would reduce the anorectic effect of melanocortin signaling in appetite control. This work inspires future studies into the relationship between progesterone- and serotonin-induced HPA axis activation, as well as mechanisms regulating hypothalamic POMC expression during pregnancy.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.L., E.R., and M.K.-W. drafted the manuscript; M.L., E.R., and M.K.-W. edited and interpreted the results of the experiments; M.L. and M.K.-W. prepared the figures; D.P., and M.K.-W. analyzed the data; M.L., E.R., D.P., and M.K.-W. inter- preted the results of the experiments; M.L. and M.K.-W. prepared the figures; M.L. and D.P. drafted the manuscript; M.L., E.R., and M.K.-W. edited and revised the manuscript; M.L., E.R., D.P., and M.K.-W. approved the final version of the manuscript.

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