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Expression of Organic Anion Transporters 1 and 3 in the Ovine Fetal Brain During the Latter Half of Gestation

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Abstract

Development and maturation of the fetal brain is critical for homeostasis *in utero*, responsiveness to fetal stress and, in ruminants, control of the timing of birth. In the sheep, as in the human, the placenta secretes estrogen and other signaling molecules into both the fetal and maternal blood, molecules whose entry or exit across the blood-brain barrier is likely to be facilitated by transporters. The purpose of this study was to test the hypothesis that the ovine fetal brain expresses organic anion transporters, and that the expression of these transporters varies as a function of brain region and fetal gestational age. Brains and pituitaries were collected at the time of sacrifice from fetal and newborn sheep at 80, 100, 120, 130, 145 days gestation and on the first day of postnatal life (parturition in sheep is at approximately 147 days gestation). Hypothalamus, medullary brainstem, cerebellum, and pituitary were processed for mRNA extraction and synthesis of cDNA (4–5/group). Real-time PCR analysis of OAT1 and OAT3 expression revealed significant expression of both genes in all of the tissues tested. In hypothalamus and cerebellum, there were statistically significant increases in the expression of one or both genes towards the end of gestation. In medullary brainstem and pituitary, the levels of expression were relatively unchanged as there were no statistically significant changes with developmental age. We conclude that the ovine fetal brain expresses both OAT1 and OAT3, that the pattern of expression suggests an increasing role for these transporters in the physiology of the developing fetal brain as the fetus nears the time of spontaneous parturition.

The mammalian fetus *in utero* develops in an estrogen-rich environment. Estrogens are involved in the triggering of labor and delivery, in the maintenance of uterine blood flow sufficient to meet the demands of the growing fetus, and estrogens are important neuroendocrine modulators of the fetal stress response. The majority of estrogen circulating in fetal plasma is sulfoconjugated. Estrone-3-sulfate and estradiol-3-sulfate circulate in high concentrations and are either excreted or deconjugated at sites of hormone action. The fetal brain takes up and deconjugates sulfoconjugated estrogens, making the unconjugated estrogen available for binding to the estrogen receptor and ultimate biological activity.

We have hypothesized that, because estrone-3-sulfate can cross the blood-brain barrier -- albeit at a lower rate than for estrone -- the fetal brain is likely to express a transporter that is known to transport sulfoconjugated steroids. In the present study, we queried cDNA prepared from brain regions collected from fetal sheep at time points throughout the latter half of gestation. We hypothesized that the fetal brain expresses members of the Organic

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Anion Transporter family. Specifically, we tested for expression of OAT3, which is known to transport estrone-3-sulfate across epithelia. We also tested for the expression of OAT1, the so-called para-amino hippurate (PAH) transporter of the kidney.

The experiments were approved by the University of Florida Animal Care and Use Committee and were performed in accordance with the American Physiological Society Guiding Principles for Research Involving Animals and Human Beings (1). Timed-dated (80, 100, 120, 130, or 145 days gestational age (DGA), $n=4-5$ per group, term=148 days) pregnant ewes, not in labor, carrying singletons or twins (one per group) were sacrificed with 20 ml Euthasol® solution (7.8 g pentobarbital and 1g phenytoin sodium; Virbac AH, Inc; Fort Worth, TX) administered intravenously. After removal of the fetus from the uterus, fetal brain was removed, dissected, and the tissues were isolated and immediately frozen in liquid nitrogen and stored at -80°C . One day and one week postpartum animals were euthanized directly and tissues collected as above. We collected hypothalamus, pituitary, and medullary brainstem because these tissues are central to the control of fetal hypothalamus-pituitary-adrenal axis activity, responsiveness to fetal stress, and control of parturition in the sheep. We chose these brain regions plus cerebellum because they express estrogen receptors and are estrogen sensitive (2–5). The medullary brainstem tissue was sectioned ~ 1 mm rostral to the obex and at the caudal medulla-rostral spinal cord border. Whole cerebellum was detached from brainstem after sectioning the cerebellar peduncles. Pituitary was removed in its entirety after removal of the diaphragma sellae. Hypothalamus was removed as a single block of tissue, bounded on the rostral edge by the rostral edge of the optic chiasm, on the caudal side by the caudal edge of the median eminence, and on the sides by the edges of the median eminence.

Total RNA was isolated from tissues using Trizol® reagent (Invitrogen, Carlsbad, CA) according to manufacturer's directions. Total RNA was treated with DNase (Ambion DNA-free ; Ambion, Inc, Austin, TX: 2U/10 μg RNA) and RNA concentration was subsequently determined by spectrophotometry. For each sample, 2 μg of total RNA was reverse transcribed using the High Capacity cDNA kit (Applied Biosystems, Foster City, CA), according to manufacturer's instructions. The resulting cDNA was used in real-time PCR reactions for OAT1 or OAT3 (100ng), and 18S ribosomal RNA (0.1ng) using TaqMan® Universal PCR Master Mix according to manufacturer's instructions (Applied Biosystems, Foster City, CA) and probes/primers designed using Primer Express (Applied Biosystems) as previously described (6). All reactions were run in triplicate in optical grade 96-well plates and caps on a ABI 7000 thermocycler (Applied Biosystems, Foster City, CA). Relative expression levels were calculated by determining the difference in cycle number (ΔC_t) between the OAT1 or OAT3 samples and the corresponding ribosomal RNA samples. Mean ΔC_t 's were calculated in triplicate, and for all samples contained in a single sample age group. ΔC_t values were adjusted by subtracting the reference value of the 80-day samples, resulting in a $\Delta\Delta C_t$ value. Fold change expression was calculated by using $2^{-\Delta\Delta C_t}$ to normalize numbers to the 80-day age group (7).

mRNA abundance was analyzed as values of ΔC_t to avoid heteroscedasticity produced by calculation of fold change ($2^{-\Delta\Delta C_t}$). Values of C_t for OAT1 in cerebellum were further corrected for heteroscedasticity by logarithmic transformation. Overall changes in mRNA abundance and differences among tissues were assessed using one-way ANOVA. Individual groups were compared using Duncan's multiple range test (8). Statistical tests were performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

OAT1 and OAT3 mRNA were found in pituitary and all brain regions tested (hypothalamus, cerebellum, and medullary brainstem) throughout the latter half of gestation in the sheep fetus (Figure 1). The ontogenetic pattern of expression for both transporters increased at the

end of gestation in hypothalamus and cerebellum but did not change significantly in medullary brainstem or pituitary. In hypothalamus, OAT1 expression changed significantly as a function of fetal gestational age ($p < 0.001$), and pairwise comparison of the individual ages revealed a progressive increase in expression with highest levels of expression measured in the postnatal ages (Figure 1, panel A). OAT3 expression also changed significantly as a function of fetal gestational age ($p < 0.05$; Figure 1, panel B), and pairwise comparison of the individual ages revealed increased expression at the end of normal gestation (145 days gestation) and in postnatal life (1 and 7 day lambs). In cerebellum, there were similar patterns of expression but these patterns were greatly exaggerated compared to the hypothalamus. OAT1 expression was quite variable, and as a result the apparent large increases were not statistically significant ($p = 0.14$; Figure 1, panel G). OAT 3 expression varied significantly with developmental age ($p < 0.001$; Figure 1, panel H). There was a statistically significant increase at 130 days and later compared to 80 days (Figure 1, panel H). Interestingly, however, this was not a progressive increase, as there was an apparent transient increase in expression at 100 days. In medullary brainstem and in pituitary, there was no statistically significant variation in OAT1 or OAT3 expression with developmental age (Figure 1, panels C, D, E, and F).

The results of this study confirm reports from other laboratories that have reported the expression of OAT1 (9–15) and OAT3 (16–24) in brain. The pattern of expression in the fetal brain is quite different from the pattern of expression in the fetal kidney (6). Both OAT1 and OAT3 expression are low throughout the majority of the latter half of gestation in the renal cortex, but increase dramatically in late gestation. We measured an approximate 100-fold increase in OAT1 and OAT3 mRNA abundance in renal cortex from fetuses at 145 days' gestation compared to 130 days' gestation (the normal timing of spontaneous parturition in sheep is approximately 147 days). In renal medulla, the pattern appeared to be similar, although not statistically significant and of lower amplitude (6). In the present study, the increase in expression of OAT1 and OAT3 in hypothalamus, pituitary, and cerebellum as the fetus neared the end of gestation started at an earlier age than the increase in expression in renal cortex. Expression in the brainstem, on the other hand, did not change as a function of fetal developmental age. These differences argue against the changes being driven by changes in circulating hormone concentrations. In the sheep fetus, there is a dramatic increase in secretion of cortisol from the adrenal cortex in the latter stages of fetal development (25;26). Because dexamethasone increases OAT1 expression in renal cortex *in vitro*, it is possible that the increased expression of OAT1 in these various tissues is in part stimulated by increased exposure to glucocorticoids (27). Similarly, it is possible that androgens and estrogens (which also increase in fetal plasma in late gestation) are involved (28). But the variation in timing of the increased expression suggests that more variables are involved. There are developmental changes in the abundance of estrogen receptors (4), androgen receptors (29), glucocorticoid receptors and mineralocorticoid receptors (30). The pattern of estrogen receptor alpha (ER- α) in cerebellum and hypothalamus appears to be quite similar to that of OAT1 in these regions, for example (4). As shown in Figure 2, *post hoc* analysis of OAT1 and ER- α mRNA abundances in cerebellum and hypothalamus reveal statistically significant linear relationships between the two variables (although the intercepts of these relationships are not similar in the two tissues). While there is some evidence that OAT1 is influenced by gonadal steroids, a relationship between ER- α and OAT1 does not prove a cause-and-effect relationship. Indeed, there is no statistically significant relationship between these variables in medullary brainstem or pituitary. Patterns of increasing expression of various genes, coincident with increasing endocrine activity, is a general physiological theme in late gestation as the fetus matures and readies for postnatal life independent of its mother. It is possible, therefore, that both ER- α and OAT1 are independently responding to the same stimulus.

The function of OAT1 and OAT3 in the brain is not clear, although it is recognized that both transporters are found in choroid plexus and are thought to be important for clearance of organic anions from the cerebrospinal fluid (9;19;24). OAT3 is also found in vascular endothelium, suggesting that the transporter is involved in transport (speculated to be transport out of the brain into blood) of organic anions across the blood-brain barrier (18). Indeed, we have been interested in the function of transporter systems that might be capable of transporting sulfoconjugated estrogens into the fetal brain, through the blood-brain barrier. We have reported that estrone-3-sulfate is taken up by the fetal brain, although at a lower rate than estrone, the unconjugated steroid (31). We suggested that there might be transport systems mediating the flux into the fetal brain (31). The presence of members of the organic anion transporter family within the brain suggests possible involvement of OAT family members, especially OAT3, known to transport sulfoconjugated steroids (32). Nevertheless, at present, there is no evidence supporting the hypothesis that OAT3 is involved in brain uptake rather than elimination of sulfoconjugated estrogens. The role of OAT1 in the brain is similarly unclear, although it seems possible that the transporter might be involved in the regulation of transmitters and neuromodulators involved in brain function or development. Indeed, ongoing studies in this laboratory reveal that blockade of this transporter family in brain with probenecid alters neuroendocrine function in the fetus (Wood and Cousins, unpublished observations).

In summary, the fetal brain expresses OAT1 and OAT3 in region-specific and age-specific patterns throughout the latter half of gestation. The general pattern of expression for both of these transporters is increased expression as the fetus nears the time of spontaneous parturition, although the specific pattern of expression is region-specific. We conclude that the capacity for transport of organic anions in the fetal brain increases as the fetus matures, although the biological significance of this increased capacity is not clear at the present time.

Research Highlights

- The fetal brain expresses Organic Anion Transporter-1 and -3 (OAT1 and OAT3) in fetal hypothalamus, pituitary, medullary brainstem, and cerebellum in region-specific and age-specific patterns throughout the latter half of gestation.
- OAT1 and OAT3 are more highly expressed as the fetus nears the time of spontaneous parturition, although the specific pattern of expression is region-specific.
- OAT1 and Estrogen Receptor Alpha mRNA abundance are highly correlated in the latter half of gestation in hypothalamus and cerebellum, but not in medullary brainstem or pituitary.

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Figure 1.

Relative abundances of OAT1 (left panels) and OAT3 (right panels) in (from top to bottom) hypothalamus (panels A and B), pituitary (panels C and D), brainstem (panels E and F), and cerebellum (panels G and H). Values are reported ($n=4-5/\text{group}$) for tissues from 80, 100, 120, 130, and 145 day gestation fetal sheep (term in this species is 147 days), as well as 1 and 7 day old lambs. Values are reported as mean values $\pm$ 1 SEM. Bars with differing superscripts are significantly different from each other ($P<0.05$ by Duncan's test). p -values reported in each panel are calculated from the respective one-way ANOVA.

Figure 2.

Linear regression analysis of OAT1 cycle threshold (Ct) *versus* ER- α Ct in hypothalamus ($\circ$) and cerebellum ($\bullet$). Both of these relationships were statistically significant. Similar regression analysis of these variables measured in brainstem ($r^2=0.012$, $n=30$) and pituitary ($r^2=0.001$, $n=27$) were not statistically significant.