Increased cerebral extracellular adenosine and decreased PGE\textsubscript{2} during ethanol-induced inhibition of FBM

CAROLE S. WATSON,\textsuperscript{1} SUSAN E. WHITE,\textsuperscript{1} J ACOBUS H. HOMAN,\textsuperscript{1} KAREN A. KIMURA,\textsuperscript{2} JAMES F. BRIEN,\textsuperscript{2} LAURENCE FRAHER,\textsuperscript{3} JOHN R. G. CHALLIS,\textsuperscript{4} AND ALAN D. BOCKING\textsuperscript{1}

\textsuperscript{1}Departments of Physiology and of Obstetrics and Gynaecology, Medical Research Council Group in Fetal and Neonatal Health and Development, \textsuperscript{2}Departments of Medicine and Biochemistry, Lawson Research Institute, University of Western Ontario, London, Ontario N6A 4V2; \textsuperscript{3}Department of Pharmacology and Toxicology, Queen's University, Kingston, Ontario K7L 3N6; and \textsuperscript{4}Department of Physiology, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Increased cerebral extracellular adenosine and decreased PGE\textsubscript{2} during ethanol-induced inhibition of FBM. J. Appl. Physiol. 86(4): 1410–1420, 1999.—Adenosine and PGE\textsubscript{2} are neuromodulators, both of which inhibit fetal breathing movements (FBM). Although circulating PGE\textsubscript{2} has been implicated as a mediator of ethanol-induced inhibition of FBM in the late-gestation ovine fetus, a role for adenosine has not been examined. The objective of this study was to determine the effect of maternal ethanol infusion on ovine fetal cerebral extracellular fluid adenosine and PGE\textsubscript{2} concentrations by using in utero microdialysis and to relate any changes to ethanol-induced inhibition of FBM. Dialysate samples were obtained from the fetal parietal cortex over 70 h after surgery to determine steady-state extracellular fluid adenosine and PGE\textsubscript{2} concentrations. On each of postoperative days 3 and 4, after a 2-h baseline period, ewes received a 1-h infusion of ethanol (1 g/kg maternal body wt) or an equivalent volume of saline, and the fetus was monitored for a further 11 h with 30-min dialysate samples collected throughout. Immediately after surgery, dialysate PGE\textsubscript{2} and adenosine concentrations were 3.7 ± 0.7 and 296 ± 127 nM, respectively. PGE\textsubscript{2} did not change over the 70 h, whereas adenosine decreased to 59 ± 14 nM (P < 0.05) at 4 h and then remained unchanged. Ethanol decreased dialysate PGE\textsubscript{2} concentration for 2 h (3.3 ± 0.3 to 1.9 ± 0.4 nM; P < 0.05) and increased adenosine concentration for 6 h (87 ± 13 to a maximum of 252 ± 59 nM, P < 0.05). Ethanol decreased FBM incidence from 47 ± 7 to 16 ± 5% (P < 0.01) for 8 h. Saline infusion did not change dialysate adenosine or PGE\textsubscript{2} concentrations or FBM incidence. These data are consistent with the hypothesis that fetal cerebral adenosine, and not PGE\textsubscript{2}, is the primary mediator of ethanol-induced inhibition of FBM at 123 days of gestation in sheep.

FETAL BREATHING MOVEMENTS (FBM) occur 30–40% of the time in the late-gestation ovine fetus in association with low-voltage electrocortical activity (LV ECoG) and eye movements (17). Ethanol is a potent inhibitor of FBM, with maternal ingestion of ethanol (0.2 g/kg maternal body wt) inhibiting FBM for at least 3 h in humans (19, 28). In sheep, a maternal intravenous infusion of ethanol (1 g/kg maternal body wt) inhibits FBM for up to 9 h (34, 41, 45). Previous studies have demonstrated that ethanol-induced inhibition of FBM in ovine fetuses >130 days of gestation is associated with increased fetal plasma and cerebrospinal fluid (CSF) concentrations of PGE\textsubscript{2} (41), which is a known inhibitor of FBM (23). However, more recent studies have shown that ethanol-induced inhibition of FBM in ovine fetuses under 130 days of gestation occurs in the absence of an increase in fetal plasma PGE\textsubscript{2} (37, 45).

Adenosine also inhibits FBM (8, 27) and the concentration of adenosine in fetal plasma, and cerebral extracellular fluid (ECF) increases during hypoxia-induced inhibition of FBM (24, 26). Adenosine has been implicated in some of the known effects of ethanol in other species (15, 16), and ethanol increases extracellular adenosine in vitro (14, 30). Despite these data, a role for adenosine in the mechanism of ethanol-induced inhibition of FBM has not been examined.

Both PGE\textsubscript{2} and adenosine are local neuromodulators in the fetal brain (29). Through the use of chronic in utero microdialysis, neurochemicals can be measured in the fetal cerebral ECF directly. A recent study using chronic microdialysis has reported variable effects of ethanol on fetal cerebral ECF concentration of PGE\textsubscript{2}, but FBM were not examined (37). Adenosine also has been measured with microdialysis in the ovine fetal cerebral ECF during acute hypoxia (26) but not during ethanol exposure. Furthermore, the effect of the surgical implantation of the microdialysis probe itself on neuromodulator concentrations has not been determined. Therefore, the objectives of this study were, first, to measure the concentrations of adenosine and PGE\textsubscript{2} in the fetal cerebral ECF after implantation of a microdialysis probe and, second, to determine the effect of maternal intravenous infusion of ethanol on fetal cerebral ECF concentration of adenosine and PGE\textsubscript{2} and to relate any changes in the concentration of these neuromodulators to the ethanol-induced inhibition of FBM. We hypothesized that maternal ethanol infusion will increase fetal cerebral ECF concentration of both adenosine and PGE\textsubscript{2} in association with an inhibition of FBM.

METHODS

Surgical procedure. Surgery was performed on 14 time-dated mixed-breed pregnant sheep at 120 days of gestation. Anesthesia was induced with intravenous thiopental sodium.
(15 mg/kg maternal body wt; Abbott Laboratories, Montreal, PQ) and maintained with inhaled 1.5% halothane (Halocarbon Laboratories, Hackensack, N J ) in 98.5% oxygen. Under sterile conditions, a midline incision was made in the ewe’s abdomen, and the fetal head and neck were exteriorized through an incision in the uterus. Polyvinyl catheters (V4, Bolab, Lake Havasu City, AZ) were placed in a fetal carotid artery, a fetal jugular vein, and the fetal trachea. Stainless steel electrodes (Cooner, Chatsworth, CA) were placed over the dura of the fetal parietal cortex through burr holes in the skull 10 mm on either side of the midline for recording of the electrocorticogram (ECoG). The electrodes were secured with plastic disks attached to the skull with cyanoacrylate glue (Krazy Glue, Bordon, Willowdale, ON). Electrodes were placed in the inner and outer canthi of one eye for recording of the fetal electrooculogram (EOG), with a common ground wire sewn into the inner surface of the scalp. A polyvinyl catheter (V1, Bolab) was sutured to the fetal skin for recording amniotic fluid pressure.

The fetal head was then placed in a Kopf 1540-A stereotaxic frame with a custom-made nose clamp (David Kopf Instruments, Tujunga, CA). A microdialysis probe (CMA/12; 14-mm shaft length, 2-mm membrane length, and 0.5-mm diameter; Chromatography Sciences, Montreal, PQ) was placed through a guide cannula (CMA/12; Chromatography Sciences) into the rostral parietal cortex through a vertical hole drilled 10 mm right of the sagittal suture at the level of the supramastoid foramen. Dental pins (Patterson Dental, London, ON) were screwed into the skull around the insertion site, and the probe was cemented in place to the dental pins with dental acrylic (Patterson Dental). Before the implantation, the microdialysis probe was perfused with sterilized, degassed artificial CSF (aCSF; 134 mM NaCl, 2 mM KCl, 1.3 mM MgSO4, 1.25 mM KH2PO4, 17 mM NaHCO3, 2.5 mM CaCl2, and 10 mM glucose) at 2 µl/min with its 1-m inlet and 1-m outlet tubings attached (Chromatography Sciences). The inlet and outlet tubings were protected from kinking by high-pressure tubing (Medex, Hilliard, OH), and a plastic cover was placed over the dental acrylic and sutured to the closed scalp to protect the probe and hold the tubing vertical at the insertion site (35).

The fetus was then returned to the uterus; the catheters, electrodes, and microdialysis probe tubing were exteriorized through the mother’s flank; and the uterus and abdomen were closed in layers. Polyvinyl catheters (V1, Bolab) were placed 20 cm into a maternal femoral artery and vein. Oxytocycline (1,600 µg; Liquomycin, Ragar/STB, London, UK) was given intramuscularly to the ewe at the time of surgery, and penicillin G sodium (1,000,000 IU) was infused into the fetal jugular vein and amniotic fluid at the time of surgery and daily for 2 days. The ewes were housed individually in metabolic cages with access to food and water ad libitum.

Collection of microdialysis samples after surgery. As soon as the ewe was breathing spontaneously and placed in a metabolic cage after surgery, the microdialysis inlet tubing was reconnected to the Harvard pump and the probe perfused with aCSF (containing the adenosine receptor blockers diprydamole (10 µM), lidoflazine (5 µM), and S-(p-nitrobenzyl)-6-thioinosine (10 µM); (26)) at 2 µl/min. A freeze pack containing each microdialysis collection tube was connected to the ewe’s back, and the tip of the outlet tubing was placed inside the tube. After at least 1 h of perfusion, 30-min samples (total volume = 60 µl) were collected at 2, 4, 8, 24, 48, and 70 h after the termination of the halothane anesthesia used during the surgery. After the 4-h sample, the Harvard pump was turned off and the tubing was disconnected. Before each subsequent sample, the tubing was reconnected, and the probe was perfused for at least 1 h before sample collection. A 7.5-min lag time before sample collection was allowed to account for the flow time from the probe membrane to the tip of the outlet tubing (dead space in the 1-m outlet tubing and probe outlet = 15 µl). All samples were immediately frozen at −70°C until analyzed. Fetal and maternal arterial blood samples (1 ml) were collected for the analysis of blood gases, pH, and glucose and lactate concentrations at 15 min into each microdialysate collection time period.

Experimental protocol. The experimental protocol was approved by the University of Western Ontario and Lawson Research Institute Animal Care Committees and was in compliance with the guidelines of the Canadian Council on Animal Care. Ten of the fourteen sheep had patent microdialysis probes on day 3, and therefore experiments were performed in these animals only. Each study consisted of a 2-h baseline period beginning between 7:00 AM and 9:00 AM, followed by a 1-h maternal intravenous infusion of either ethanol (40% vol/vol solution, Liquor Control Board of Ontario) at a dose of 1 g/kg maternal body wt or an equivalent volume of saline. The fetuses were then monitored for a further 11 h. One ethanol and one saline experiment were performed in seven of the animals on concurrent days in random order. One ethanol experiment only was performed in three sheep because the microdialysis probe stopped working after that experiment. Throughout the 14 h of each experiment, 30-min microdialysate samples (2 µl/min) were collected continuously as described in Collection of microdialysate samples after surgery, allowing the 7.5-min lag time to account for the 15 µl of dead space between the microdialysis membrane and the tip of the 1-m outlet tubing, and were immediately frozen at −70°C until analyzed. Fetal and maternal arterial blood samples (1 ml) were obtained at the start of the 2-h baseline period, at the start (0 h) and end (+1 h) of the infusion period and every 2–4 h during the recovery period for analysis of blood gases, pH, and glucose and lactate concentrations. In two animals, 0.2-ml aliquots of fetal and maternal arterial blood were collected at the same time points for the determination of blood ethanol concentrations. In one of these two animals, dialysate samples collected at the same time points as the blood samples were used for analysis of dialysate ethanol concentration.

At the conclusion of the experiments, the ewe and fetus were euthanized with an overdose of intravenous pentobarbital sodium (Euthanyl, MTC Pharmaceuticals, Cambridge, ON). The fetus was quickly removed from the uterus, and the fetal brain was perfused in situ with ice-cold saline followed by 10% buffered Formalin. The brains were cut in coronal sections of 5-µm thickness followed by staining with hematoxylin and eosin for determination of the membrane placement. In two fetuses, the microdialysis probe was perfused with cresyl red dye at a rate of 20 µl/min, which was rapid enough to rupture the dialysis membrane.

Data collection. Continuous recordings of ECoG, EOG, fetal tracheal and arterial pressures, amniotic fluid pressure, and fetal heart rate (FHR) were obtained throughout each experiment and displayed on a polygraph. Pressures were recorded with Statham pressure transducers (P-23ID, Viggo-Spex-tramed, Oxnard, CA) and direct-current pressure amplifiers (model 7P1, Astro-Med, Grass Div., Boucherville, PQ). FBM was defined as repeated negative deflections in tracheal pressure of >2 mmHg, which lasted for at least 30 s (10) because 2 mmHg is the smallest change in tracheal pressure that can be consistently determined through visual inspection of the polygraph recording. Mean fetal blood pressure (MAP) was calculated as the diastolic pressure + 0.4(syst-
tolic – diastolic pressure) after the subtraction of amniotic fluid pressure from the diastolic and systolic pressures. FHR was measured by a cardiotachometer (model 7P44B, Grass) triggered from the arterial pulse pressure. ECoG was recorded by using an alternating-current electroencephalogram preamplifier (model 7P511, Grass). For each animal, a minimum level for high-voltage ECoG (range 55–125 µV) and a maximum level for low-voltage ECoG (range 26–83 µV) were determined during the baseline period to allow for interanimal variation. The same levels could not be used for all animals because each animal displayed different thresholds for low and high voltage. If the voltage fell between the two limits, the ECoG was described as indeterminate. For the ECoG voltage to be considered changed, a new voltage level had to be present for at least 30 s. EOG was also recorded continuously by using an alternating-current electroencephalogram preamplifier. The presence of eye movement activity was determined through visual inspection of the record, and, for an episode of eye movements to be identified, electrical activity must have been present for at least 30 s.

Determination of blood gases, pH, glucose, and lactate. Blood samples were collected in ice-cold heparinized syringes and immediately placed on ice. Arterial PaO2, PaCO2, and pH were measured by using an ABL 500 blood gas analyzer (Radiometer, Copenhagen, Denmark) at 37°C and corrected for a fetal temperature of 39.5°C. Arterial oxygen saturation (SaO2) and Hb concentration were measured with an OSM 3 Hemoximeter (Radiometer). Blood glucose and lactate concentrations were measured by the glucose and lactate oxidase methods by using a glucose and lactate analyzer (model 2300 Stat Plus, Yellow Springs Instruments).

Determination of blood and dialysate ethanol concentrations. Blood and dialysate ethanol concentrations were measured by an established method involving gas-liquid chromatography with headspace-gas analysis (42). A 100-µl aliquot of blood or a 50-µl aliquot of dialysate, diluted to 100 µl with aCSF, were mixed with 900 µl of an ice-cold solution containing 875 µl perchloric acid (34 mg/ml) in saline and 25 µl aqueous 1-propanol (3 mg/ml) as the internal standard. This mixture was then centrifuged at 13,000 g for 30 s, and 200 µl of supernatant were placed in a sealed hypovial and frozen at −70°C until analyzed. The recovery, within-day coefficient of variation and lower limit of quantifiable sensitivity for the method were 99.5%, 6.8%, and 0.005 mg/ml, respectively.

In view of the fact that the microdialysis membrane can only sample a percentage of all the molecules in a particular extracellular space (4), in vitro recovery of ethanol was determined to provide a measurement of the cerebral ECF ethanol concentration. A microdialysis probe was placed in solutions containing varying concentrations of ethanol from 0 to 1.6 mg/ml (based on expected fetal blood ethanol concentrations) at 39.5°C and perfused with aCSF at 2 µl/min. Microdialysate samples (50 µl) were collected, diluted, and analyzed for ethanol concentration as described above. The in vitro recovery percentage of ethanol was then calculated as the ethanol concentration in the collected sample divided by the known ethanol concentration in the fluid surrounding the membrane, multiplied by 100.

Determination of dialysate adenosine and PGE2 concentrations. Dialysate adenosine concentrations were determined by high-pressure liquid chromatography. A 30-µl aliquot of each sample was injected (Waters 712 WISP) onto a 4.6 × 25-cm column (Novapack C18) by using a mobile phase consisting of 95% 20 mM KH2PO4 (pH 5.7)-5% methanol buffer (vol/vol) at a flow rate of 1 ml/min. An ultraviolet detector was set at 254 nm to quantitate adenosine (Waters 481). The adenosine chromatographic signal was identified by retention time with a reference standard of 100 nM, and the adenosine concentration was calculated for each sample by interpolation of the area of the adenosine chromatographic signal on an aqueous adenosine standard curve. The minimum measurable concentration of adenosine was 10 nM (21). Dialysate PGE2 concentration was determined by using a radioimmunoassay, on the basis of a previously described method (31) with no preassay extraction (37). For the samples taken after surgery, 20 µl of dialysate from each sample were assayed for PGE2. For the experimental samples, 20 µl from each of the two samples collected each hour were pooled to have 40 µl for PGE2 analysis. The dialysate samples from only 10 of the 14 animals studied after surgery and from 8 of the 10 ethanol infusion experiments and 6 of the 7 saline infusion experiments were included for PGE2 analysis because of unforeseen sample volume loss. All PGE2 measurements were performed in one assay. The intra-assay coefficient of variation was 4.5%, and the lower limit of sensitivity was 10 pg/sample. The specific PGE2 antibody for this assay was kindly supplied by Dr. T. G. Kennedy (University of Western Ontario).

Data analysis. The data are presented as group means ± SE. Statistical analysis was performed by using two-way ANOVA for repeated measures, with post hoc Dunnett's t-tests where significance was indicated across time in each group. Student's t-tests with a pooled variance were performed where significance was indicated between ethanol and saline groups for each time point. Post hoc analysis of area under the curve was also conducted for the experimental concentrations of adenosine and PGE2. For the concentration of adenosine, the mean area under the curve was calculated for the time periods 0–8 h and 0–5.5 h and was compared between the ethanol and saline groups by a Student's t-test. For the concentration of PGE2, the mean area under the curve was calculated for the time periods 0–8 h and 0–2 h. For both neuromodulators, these time periods were chosen on the basis of the results of the ANOVA and post hoc Dunnett's and t-tests. Two sets of data were considered to be statistically different when P < 0.05.

### RESULTS

Blood gases, pH, and glucose, lactate, and Hb concentrations after surgery. At 2 h after surgery, fetal arterial PaO2 was 26.1 ± 0.8 Torr, SaO2 was 78.2 ± 2.2%, and pH was 7.39 ± 0.01. These values decreased to 23.7 ± 0.8 Torr (P < 0.05), 68.9 ± 2.2% (P < 0.05), and 7.36 ± 0.01 (P < 0.01), respectively, by 8 h, with no further changes over 70 h. Fetal arterial PaCO2 did not change significantly from 49.9 ± 1.2 Torr 2 h after surgery until an increase to 55.3 ± 1.3 Torr (P < 0.01) at 70 h. Fetal arterial lactate concentration was 1.4 ± 0.2 mmol/l at 2 h after surgery and decreased to 0.9 ± 0.1 mmol/l (P < 0.01) by 24 h, with no further change over 70 h. Fetal arterial Hb was 11.6 ± 0.2 g/100 g at 2 h after surgery and decreased to 10.7 ± 0.3 g/100 g at 8 h, with no further change over 70 h (Table 1). Fetal arterial glucose concentration was 1.0 ± 0.1 mmol/l at 2 h after surgery, with no significant change over 70 h.

Dialysate concentrations of adenosine and PGE2 after surgery. The concentration of adenosine in the dialysate at 2 h after surgery was 296 ± 127 nM. Dialysate adenosine concentration decreased to 59 ± 14 nM (P < 0.05) at 4 h with no further change over 70 h. At 2 h
Table 1. Fetal arterial blood gases, pH, base excess, lactate, and Hb concentrations after surgery

<table>
<thead>
<tr>
<th>Time after surgery, h</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
<th>48</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td>PO₂, Torr</td>
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<td>23.7±0.8†</td>
<td>24.1±1.0†</td>
<td>23.2±0.7†</td>
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<td>SaO₂, %</td>
<td>78.2±2.2</td>
<td>74.6±2.0</td>
<td>68.9±2.2†</td>
<td>72.6±2.3†</td>
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</tr>
<tr>
<td>BE, mmol/l</td>
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<td>1.7±0.3*</td>
<td>1.8±0.4*</td>
<td>2.4±0.4*</td>
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<td>Lactate, mmol/l</td>
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<td>1.8±0.2</td>
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<td>Hb, ng/100 g</td>
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<td>11.2±0.3</td>
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<td>10.2±0.2*</td>
<td>9.7±0.3*</td>
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</tr>
</tbody>
</table>

Values are means ± SE. SaO₂, arterial O₂ saturation; BE, base excess. *P < 0.01. †P < 0.05 compared with 2 h.

Table 1. Fetal arterial blood gases, pH, base excess, lactate, and Hb concentrations after surgery

After surgery, dialysate PGE₂ concentration was 3.72 ± 0.70 nM, with no change over 70 h (Fig. 1).

Fetal arterial blood gases, pH, and glucose, lactate, and Hb concentrations. During the 2-h baseline period, fetal arterial PO₂, SaO₂, and Pco₂ were 22.4 ± 1.3 Torr, 65.9 ± 3.5%, and 56.1 ± 1.5 Torr for the ethanol group and 20.6 ± 0.9 Torr, 58.8 ± 3.0%, and 54.8 ± 1.0 Torr for the saline group, respectively. At the end of the 1-h maternal ethanol infusion, PO₂ and SaO₂ increased to 24.8 ± 1.6 Torr (P < 0.05) and 70.6 ± 3.4% (P < 0.05), respectively, and Pco₂ decreased to 50.4 ± 1.4 Torr (P < 0.01) at 7 h (Table 2). Before the maternal infusions, pH, base excess (BE), and lactate concentration were 7.36 ± 0.01, 1.44 ± 0.6 mmol/l, and 1.0 ± 0.1 mmol/l in the ethanol group and 7.35 ± 0.00, 3.7 ± 0.3 mmol/l, and 1.1 ± 0.1 mmol/l in the saline group, respectively. After the ethanol infusion, pH increased to 7.38 ± 0.01 (P < 0.05) at 7 h; BE increased to 4.9 ± 0.7 mmol/l (P < 0.05) at 3 h; and lactate concentration increased to 1.6 ± 0.2 mmol/l (P < 0.01) at 12 h (Table 2). Fetal arterial blood glucose concentration was 1.0 ± 0.0 and 0.9 ± 0.2 mmol/l before the infusions in the ethanol and saline groups, respectively. Glucose concentration decreased after the ethanol infusion to 0.9 ± 0.0 mmol/l (P < 0.05) at 3 h (Table 2). There were no changes in fetal PO₂, SaO₂, Pco₂, pH, BE, or lactate or glucose concentrations with the infusion of saline (Table 2). Baseline fetal arterial Hb was 9.0 ± 0.3 and 9.2 ± 0.2 ng/100 g in the ethanol and saline groups, respectively, followed by no change with either infusion.

Blood and dialysate ethanol concentrations. Fetal arterial blood ethanol concentration (BEC) was maximal (1.4 mg/ml) at the end of the 1-h maternal ethanol infusion, decreasing to 0.2 mg/ml at 12 h (n = 2). Maternal arterial BEC was also maximal (1.7 mg/ml) at the end of the ethanol infusion and was 0.1 mg/ml at 12 h (Fig. 2). Fetal cerebral dialysate concentration of ethanol was maximal (0.58 mg/ml) during the sampling time between 0.5 and 1 h (the second half of the 1-h maternal ethanol infusion) and was 0.06 mg/ml during the sampling time between 11 and 11.5 h. The microdialysis probe in vitro recovery for ethanol was an average of 21.3% (range 13–25%). When the dialysate ethanol concentration data were corrected for microdialysis probe recovery, the concentration of ethanol in the fetal cerebral ECF was maximal (2.7 mg/ml) between 0.5 and 1 h and declined to 0.3 mg/ml by 11.5 h (Fig. 2).

MAP and FHR. Fetal MAP before the infusions was 40.8 ± 1.1 and 42.0 ± 0.9 Torr in the ethanol and saline groups, respectively, with no changes with either treatment. FHR before the infusions was 189 ± 5 and 185 ± 8 beats/min in the ethanol and saline groups, respectively. FHR decreased during the ethanol infusion to 180 ± 3 beats/min (P < 0.01) and remained significantly decreased for 5 h. There was no change in FHR with the saline infusion.

Dialysate concentration of adenosine and PGE₂. Ethanol and saline infusions were performed on concurrent
days, in random order. There was no difference in the baseline concentration of adenosine or PGE\textsubscript{2} in the dialysate between experiments performed on day 1 (adenosine: 106 ± 37 nM, n = 10; PGE\textsubscript{2}: 3.2 ± 0.3 nM, n = 10) and those performed on day 2 (adenosine: 105 ± 45 nM, n = 7; PGE\textsubscript{2}: 3.8 ± 0.4 nM, n = 7).

Dialysate adenosine concentration was 87 ± 13 and 120 ± 55 nM during the 2-h baseline period in the ethanol and saline groups, respectively. Dialysate adenosine concentration increased during the infusion of ethanol to a maximum of 252 ± 59 nM (P < 0.05), representing an increase of 279 ± 128% above baseline. Dialysate adenosine concentration remained significantly elevated for 4.5 h after the ethanol infusion (P < 0.05), on the basis of post hoc analysis with Dunnett’s t-test (Fig. 3). When area under the curve analysis was performed, the concentration of adenosine in the dialysate was significantly elevated for 7 h after the infusion of ethanol, compared with the infusion of saline (P < 0.05; Table 3). There were no changes in the dialysate concentration of adenosine during or after the infusion of saline (Fig. 3).

Dialysate PGE\textsubscript{2} concentration was 3.3 ± 0.3 and 4.0 ± 0.5 nM during the 2-h baseline period in the ethanol and saline groups, respectively. During the ethanol infusion, dialysate PGE\textsubscript{2} concentration decreased to 1.9 ± 0.4 nM (68 ± 13% of baseline; P < 0.05) and remained significantly decreased for a further 1 h before returning to baseline, on the basis of both post hoc Dunnett’s t-test and area under the curve analyses. There were no changes in dialysate PGE\textsubscript{2} concentration during or after the saline infusion (Fig. 4, Table 3).

### Table 2. Fetal arterial blood gases, pH, base excess, and glucose and lactate concentrations before (0 h), at the end of (1 h), and after (3, 7, and 12 h) the maternal intravenous infusion of ethanol (1 g/kg maternal body wt) or equivalent volume of saline

<table>
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<tr>
<th>Time, h</th>
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<td></td>
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<tr>
<td>P\textsubscript{o}2, Torr</td>
<td>22.4± 1.3</td>
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<td>S\textsubscript{a}O\textsubscript{2}, %</td>
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<td>BE, mmol/l</td>
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<td>1.2± 0.1</td>
<td>1.4± 0.1</td>
<td>1.6± 0.2</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>1.0± 0.0</td>
<td>0.9± 0.0</td>
<td>0.9± 0.0</td>
<td>0.9± 0.1</td>
<td>1.0± 0.2</td>
</tr>
<tr>
<td><strong>Saline (n = 7)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P\textsubscript{o}2, Torr</td>
<td>20.6± 0.9</td>
<td>20.9± 1.1</td>
<td>19.8± 0.9</td>
<td>20.5± 0.8</td>
<td>19.0±1.0</td>
</tr>
<tr>
<td>S\textsubscript{a}O\textsubscript{2}, %</td>
<td>58.8± 3.0</td>
<td>59.5± 3.3</td>
<td>56.2± 3.5</td>
<td>58.4± 3.0</td>
<td>52.3± 4.3</td>
</tr>
<tr>
<td>P\textsubscript{CO}2, Torr</td>
<td>54.8± 1.0</td>
<td>54.6± 1.1</td>
<td>53.3± 0.8</td>
<td>53.5± 0.8</td>
<td>55.0± 0.8</td>
</tr>
<tr>
<td>pH</td>
<td>7.35± 0.00</td>
<td>7.36± 0.01</td>
<td>7.35± 0.00</td>
<td>7.34± 0.01</td>
<td>7.35± 0.01</td>
</tr>
<tr>
<td>BE, mmol/l</td>
<td>3.7± 0.3</td>
<td>3.9± 0.5</td>
<td>3.1± 0.4</td>
<td>3.2± 0.4</td>
<td>3.5± 0.4</td>
</tr>
<tr>
<td>Lactate, mmol/l</td>
<td>1.1± 0.1</td>
<td>1.2± 0.1</td>
<td>1.3± 0.1</td>
<td>1.2± 0.1</td>
<td>1.1± 0.1</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>0.9± 0.2</td>
<td>1.1± 0.1</td>
<td>1.2± 0.2</td>
<td>1.0± 0.1</td>
<td>0.8± 0.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of fetuses. *P < 0.01, †P < 0.05 compared with 0 h in same group. ‡P < 0.01, §P < 0.05, compared with saline group.

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![Fig. 2. Fetal arterial (○; n = 2 fetuses), maternal arterial (●; n = 2 ewes), and fetal cerebral dialysate (□; n = 1 fetus) concentrations of ethanol before, during, and after 1-h maternal intravenous infusion of ethanol (1 g/kg maternal body wt), indicated by hatched bar.](image)

![Fig. 3. Dialysate adenosine concentration (expressed as percentage of 2-h baseline mean) during and after 1-h maternal infusion of ethanol (solid line; n = 10 fetuses) or saline (dotted line; n = 7 fetuses), indicated by hatched bar. Values are means ± SE. *P < 0.05 compared with baseline period. †P < 0.01, ‡P < 0.05, comparison between ethanol and saline groups.](image)
FBM, ECoG, and EOG. FBM occurred 46.7 ± 6.8 and 38.6 ± 6.4% of the time during the 2-h baseline period in the ethanol and saline groups, respectively. During the ethanol infusion, FBM incidence decreased to 16.1 ± 5.2% of the time (P < 0.01) and remained significantly decreased for 7 h after the end of the infusion (Fig. 5).

Eye movements occurred 54.6 ± 3.8 and 47.9 ± 5.6% of the time during the 2-h baseline period in the ethanol and saline groups, respectively. The incidence of eye movements also decreased to 34.5 ± 4.5% of the time (P < 0.05) during the ethanol infusion and remained significantly decreased for a further 1 h. There were no changes in the incidence of FBM or eye movements during or after saline infusion (Fig. 5).

The incidence of LV ECoG during the 2-h baseline period was 55.7 ± 3.7 and 52.3 ± 4.9% for the ethanol and saline groups, respectively. LV ECoG incidence decreased during the 1-h ethanol infusion to 39.9 ± 6.7% (P < 0.01) and returned to baseline immediately after the end of the infusion. High-voltage (HV) ECoG also decreased from a baseline incidence of 27.0 ± 3.6 to 9.8 ± 2.5% (P < 0.01) during the ethanol infusion and remained significantly decreased for 3 h. In contrast, the incidence of indeterminate ECoG increased during the ethanol infusion from 13.8 ± 2.3 to 50.4 ± 7.8% (P < 0.01) and remained significantly elevated for 4 h. There were no changes in the incidences of LV, HV, or indeterminate ECoG with the saline infusion (Fig. 5).

Fig. 5. Incidence of fetal breathing movements (FBM; A) and eye movements (B) in hourly epochs before, during, and after 1-h maternal intravenous infusion of ethanol (solid lines; n = 10 fetuses) or saline (dotted lines; n = 7 fetuses), indicated by hatched bar. Values are means ± SE. **P < 0.01, *P < 0.05 compared with baseline period. †P < 0.01, †P < 0.05, comparison between ethanol and saline groups.

Table 3. Comparison of the area under the curve for the concentration of adenosine and prostaglandin E2 in the dialysate of the fetal cerebral extracellular fluid infusion of either ethanol or saline

<table>
<thead>
<tr>
<th>Time Period</th>
<th>0–5.5 h</th>
<th>0–8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Adenosine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>1,393 ± 342*</td>
<td>1,650 ± 380*</td>
</tr>
<tr>
<td>Saline</td>
<td>622 ± 73</td>
<td>810 ± 117</td>
</tr>
<tr>
<td><strong>Prostaglandin E2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol</td>
<td>297 ± 27*</td>
<td>895 ± 91</td>
</tr>
<tr>
<td>Saline</td>
<td>374 ± 34</td>
<td>1,040 ± 207</td>
</tr>
</tbody>
</table>

Values are means ± SE in arbitrary units. Time periods of 0–5.5 h and 0–8 h for adenosine and 0–2 h and 0–8 h for prostaglandin E2 were chosen based on post hoc analysis of neuromodulator concentrations and fetal breathing movement incidence. *P < 0.05 compared with saline control group.

Fig. 4. Dialysate PGE2 concentration (expressed as percentage of 2-h baseline mean) during and after 1-h maternal intravenous infusion of ethanol (solid line; n = 8 fetuses) or saline (dotted line; n = 6 fetuses), indicated by hatched bar. Values are means ± SE. *P < 0.05 compared with baseline period. †P < 0.05, comparison between ethanol and saline groups.

Fig. 6. Placement of the microdialysis probe. In the two animals in which Cressyl red dye was perfused into the dialysis membrane at a rate fast enough to rupture the membrane, the presence of dye was confirmed in the white matter of the fetal cerebral cortex. In fetal brains stained with hematoxylin and eosin, the tip of the dialysis membrane was also found to be present in the white matter of the fetal cerebral cortex.
In this study, we have utilized chronic in utero microdialysis in the ovine fetal cerebral cortex while simultaneously monitoring fetal behavioral indexes such as FBM, ECoG, and eye movements. We have shown that maternal intravenous infusion of ethanol at a dose of 1 g/kg maternal body wt leads to an increase in adenosine and a decrease in PGE2 concentration in the cerebral ECF of the late-gestation ovine fetus at a time when FBM are inhibited. We have also demonstrated that adenosine and PGE2 in the fetal cerebral ECF reach steady-state concentrations by at least 4 h after surgical microdialysis probe implantation and that the presence of a microdialysis probe in the fetal cerebral cortex has no effect on FBM, ECoG, or eye movements under control conditions or during ethanol exposure. The dose of ethanol used in this study was previously established in our laboratory as one that reproduces the effects of ethanol on human FBM (34). The maximal blood ethanol concentrations achieved in the fetal and maternal arterial blood were 1.4 and 1.7 mg/ml, respectively. For comparison purposes, the blood ethanol concentration limit to legally operate a motor vehicle in Ontario is 0.8 mg/ml. Although the amount of alcohol used in this study is equivalent to the intake of several drinks over 1 h, the effects on ovine FBM with this dose are similar to those seen in women after the intake of one to three drinks (19, 28). In addition, because of the use of a single infusion, this dose mimics a pattern of binge drinking. Although the incidence of alcoholism during pregnancy is low in the general population (1), not all women completely abstain from alcohol throughout their pregnancies (44). Therefore, it is important to understand the effects of low-to-moderate intake of ethanol on the developing fetus, and FBM incidence is one of the most sensitive indexes of ethanol exposure.

In these experiments it is assumed that ethanol-induced changes in neuromodulator concentrations measured in the cerebral cortex are similar to those occurring in fetal brain stem areas known to affect FBM, such as the nucleus of solitary tract or nucleus parabrachialis medialis. In support of this assumption is the fact that ethanol is a polar molecule that distributes quickly into tissues and across membranes (12) and is therefore likely to affect all parts of the fetal brain equally. Preliminary measurements indicate that the concentration of ethanol in the ECF of the fetal cerebral cortex is similar to that found in fetal arterial blood. Adenosine has been shown to be present in the ovine fetal brain stem and midbrain ECF under basal conditions in concentrations similar to those found in the cerebral cortex in this study (26). In addition, hypoxia-induced inhibition of FBM is associated with an increase in the concentration of adenosine in the fetal brain stem/midbrain ECF of a similar magnitude as seen in this study (26).

Microdialysis does not allow the measurement of absolute concentrations of neurochemicals directly, because the interstitial concentration of the molecules does not equilibrate rapidly with the large volume of the microdialysis perfusate (4). Adenosine is rapidly removed from the ECF because of the presence of efficient reuptake transporters in cellular membranes, and, therefore, adenosine reuptake blockers were included in the perfusate (3, 26). In vitro calculations were not made of the membrane diffusion capacities for adenosine or PGE2 into the perfusate because transport characteristics of the microdialysis membrane in vitro differ from those in vivo (4). Therefore, the focus of this study was on determining relative changes in dialysate concentrations of adenosine and PGE2 with ethanol exposure. However, in measuring ethanol concentrations in the dialysate, we did determine an in vitro recovery of the microdialysis membrane for ethanol. On the basis of the data for two animals, the maximal concentration of ethanol in the cerebral ECF, after correction for the microdialysis recovery, was higher than that found in the fetal blood. In the hours after the maternal ethanol infusion, the calculated concentration of ethanol in the fetal cerebral ECF was similar to
that found in the fetal and maternal blood, which is in keeping with the ability of ethanol to diffuse freely through biological membranes and into the interstitial fluid of the fetal brain (12).

It is also important to recognize that implantation of the microdialysis probe in the brain itself is associated with damage-induced changes in the immediate microenvironment. Local cerebral glucose metabolism is increased and local cerebral blood flow is decreased at 3 h after probe implantation in adult rats, but both glucose metabolism and blood flow return to control by 24 h (7). Furthermore, the blood-brain barrier has been shown to be intact by 30 min after probe implantation (6). Gliosis at the membrane does not become apparent until at least 3 days after implantation in the rat (5). Although these effects have not been examined in the ovine fetus, it is likely that the microenvironment surrounding the microdialysis membrane in our experiments had returned to normal and gliosis at the membrane was minimal because our studies were performed 3 days after surgery. As a partial confirmation of the lack of an effect of gliosis on the membrane diffusion at the time of the experiments, baseline concentrations of adenosine and PGE$_2$ in the dialysate did not differ between the 2 experimental days.

Despite the initial damage induced by surgical implantation of the microdialysis probe, ECF adenosine reached steady-state levels by 4 h after surgery, and this time course parallels that seen in awake adult rats after surgical microdialysis probe implantation (3). This increase in ECF adenosine is likely related to the local tissue damage, including decreased blood flow, increased glucose metabolism, and direct cellular damage. In contrast, the concentration of PGE$_2$ in the dialysate did not change in the samples collected during the 70 h after surgery, indicating that fetal cerebral ECF PGE$_2$ had reached a steady state by 2 h after surgery. Local production of prostaglandins has been implicated in the changes that occur after brain injury, as described above, because one of the first events to occur with cellular injury is the disruption of cell membranes and release of fatty acids, which can then be metabolized to eicosanoids (33, 49). These data demonstrate the important fact that, during the use of chronic in utero microdialysis in the ovine fetal brain, release and reuptake of the neuromodulators adenosine and PGE$_2$ are at a steady state as early as 4 h after microdialysis probe implantation. In addition, the incidence of FBM, eye movements, and LV, HV, and indeterminate ECoG during the control periods in these experiments is consistent with previous studies (17, 34, 41, 45), indicating that the presence of a microdialysis probe in the fetal cerebral cortex has no effect on behavioral states in fetal sheep.

FBM were inhibited for 8 h by ethanol exposure, as seen in previous studies (34, 41, 45). Concentration of adenosine in the fetal cerebral ECF was also increased for the same time period after the infusion of ethanol, compared with saline infusion. Adenosine is known to inhibit FBM (8, 27), whereas adenosine-receptor blockade stimulates FBM (2, 27). Adenosine release is thought to mediate the hypoxia-induced inhibition of FBM in sheep (24, 26). Because of the variability in the concentration of adenosine and in the incidence of FBM, both between animals and over time for each animal, a significant negative correlation between increasing adenosine and decreasing FBM could not be established. However, on the basis of the temporal relationship between inhibition of FBM incidence and increased ECF concentration of adenosine in the fetal brain, our study is consistent with adenosine's playing a role in ethanol-induced inhibition of FBM. However, it will be important to determine the concentration of adenosine in other areas of the fetal brain such as the pons and/or medulla to establish that the changes demonstrated in this study are consistent throughout the brain.

Initially during the 1-h maternal ethanol infusion, fetal cerebral ECF concentration of adenosine increased nearly threefold from baseline. Adenosine levels in the dialysate then decreased slightly, although they remained elevated by approximately twofold for a further 7 h. Reynolds and Brien (36) demonstrated a similar initial effect of ethanol on adenosine efflux in fetal guinea pig hippocampal slices in vitro. They found that exposure to 48 mM ethanol increased adenosine efflux maximally during the first 10 min of exposure, followed by a return to spontaneous efflux levels.

The mechanism for the increased fetal cerebral ECF adenosine concentration with ethanol exposure is not known. Ethanol increases extracellular adenosine through several mechanisms, including stimulating the transporter protein that moves adenosine out of cells (14), inhibiting the reuptake of adenosine (30), and via the metabolism of ethanol itself (13). Ethanol is oxidized to acetate via acetaldehyde. The subsequent biotransformation of acetate to acetyl CoA requires ATP, the breakdown of which results in the production of adenosine (13). Although some of the effects of ethanol are thought to be mediated through this pathway, the metabolism of acetate in the fetal brain has not been examined. It is possible that the initial large increase in the concentration of extracellular adenosine is related to an initial release of adenosine or AMP from the cells. The subsequent prolonged increase in extracellular adenosine over 7 h may then be related to a decrease in reuptake or to the metabolism of acetate, because adenosine and AMP are depleted in the cells over a period of hours. During acute hypoxia, the initial increase in adenosine seen in the fetal cerebral ECF has been shown to be dependent on the hydrolysis of extracellular AMP by ectonucleotidase (25).

In contrast to adenosine, the concentration of PGE$_2$ in the fetal cerebral ECF decreased to 68% of baseline during the 1-h maternal ethanol infusion and remained decreased for a further 1 h after cessation of the infusion. The concentration of PGE$_2$ in the dialysate then returned to baseline for the remainder of the experiment, despite the continued inhibition of FBM. Because of the variability in the concentration of PGE$_2$ and in the incidence of FBM, both between animals and over time for each animal, a significant correlation
between PGE$_2$ and FBM could not be established. In a recent study (37), the same ethanol infusion regimen was shown to have a variable effect on the concentration of PGE$_2$ in dialysate obtained from the parasagittal parietal cortex of the late-gestation ovine fetus. Most fetuses in that study showed an increase in dialysate PGE$_2$ with ethanol exposure, but some fetuses demonstrated an apparent decrease. It would be of interest to obtain microdialysate samples from other regions of the fetal brain to determine whether the ethanol-induced decrease in cerebral ECF PGE$_2$ concentration seen in these studies is global. However, these data are in agreement with the study of Sinervo et al. (39), in which efflux of PGE$_2$ was shown to decrease in late-gestation ovine fetal brain stem slices during in vitro ethanol exposure.

Previous studies using fetuses at this gestational age (37, 45) have shown no effect of ethanol on fetal plasma concentration of PGE$_2$, despite an inhibition of FBM. This is in contrast to fetuses older than 130 days of gestation, in which an increase in both fetal plasma and CSF concentrations of PGE$_2$ occurs with this ethanol infusion, in association with decreased FBM incidence (41). The increase in circulating PGE$_2$ concentrations in those studies is believed to originate from the placenta (11). We have suggested that this differential age effect may be related to the increase in placental PGH synthase type II, which occurs after 130 days of gestation (20, 48). Experiments in the present study were performed at 123 days of gestation to determine whether there are local changes in the concentration of PGE$_2$ in the fetal brain during ethanol-induced inhibition of FBM because we would not anticipate changes in circulating concentrations of PGE$_2$. Maternal ethanol infusion has also been shown to have no effect on either PGE$_2$ concentrations or incidence of FBM in ovine fetuses at 90 days of gestation (9).

The mechanism for decreased fetal cerebral ECF PGE$_2$ concentration with ethanol is unknown. PGE$_2$ within the ECF of the fetal brain could originate locally (32), or it could arise from a peripheral source, such as the placenta, because PGE$_3$ is known to cross the ovine fetal blood-brain barrier (22). However, it is most likely that the decrease in PGE$_2$ concentration in the cerebral ECF seen in our study with ethanol infusion occurs as a result of a change in the production or metabolism of PGE$_2$ in the fetal brain itself.

The decrease in the incidence of eye movements and LV ECoG produced by maternal ethanol infusion in these experiments is consistent with that of previous studies (34, 41, 45). LV and HV ECoG are replaced by an ECoG state that we have named indeterminate, because the voltage falls between that of LV and HV ECoG. The physiological significance of this ECoG state is unknown, and it would be of interest to determine the power spectrum of this ECoG state during ethanol exposure. It is important to note that ECoG state and FBM are affected by ethanol via separate sites in the fetal brain, because the inhibitions of FBM and LV ECoG occur over different time periods. Exogenous adenosine inhibits eye movements for 4 h and LV ECoG for 1 h during an 8-h infusion, similar to the effect of ethanol seen in our study (27). Exogenous PGE$_2$ has no effect on fetal ECoG state (23, 46), whereas ethanol-induced inhibition of both eye movements and LV ECoG cannot be blocked by indomethacin (40). The power spectrum of the fetal ECoG has not been examined during infusion of either PGE$_2$ or adenosine. Overall, the present data indicate that ethanol-induced inhibition of both eye movements and LV ECoG in the ovine fetus may be due to an increase in the concentration of extracellular adenosine and not PGE$_2$ in the fetal brain.

At the end of the ethanol infusion, fetal arterial PO$_2$ and $S_aO_2$ were both elevated, and there was a delayed increase in arterial pH, which was associated with an increase in BE and lactate concentration and a decrease in PCO$_2$. Fetal arterial glucose concentration decreased after ethanol infusion. In previous studies in which this dose of ethanol was used (34, 38, 41), a decrease in fetal arterial blood glucose concentration has been demonstrated, but no changes in fetal PO$_2$, PCO$_2$, or pH have been reported. Ethanol inhibits gluconeogenesis, which is one source of glucose in the sheep fetus (43, 47). In addition, maternal arterial glucose concentration also decreased after infusion of ethanol (2.7 ± 0.1 to 2.1 ± 0.1 mmol/l; P < 0.05), which likely resulted in a decrease in glucose transfer to the fetus. The mechanism for the increase in fetal arterial pH with ethanol is less clear. The alkalosis occurred at the same time as a decrease in PCO$_2$, and an increase in BE, indicating both a metabolic and respiratory component, possibly due to a decrease in placental blood flow during ethanol infusion (18). The increase in fetal arterial concentration of lactate may be due to an increase in the reduction of pyruvate to lactate, which occurs during the conversion of alcohol to acetaldehyde. Furthermore, the small increase in PO$_2$ after the ethanol infusion may be related to the concurrent decrease in FBM, which contributes significantly to oxygen consumption in the fetus. Fetal arterial PCO$_2$ was slightly higher under basal conditions than reported in previous studies in fetuses at this gestational age (17, 23, 34, 41). However, there was no difference in baseline PCO$_2$ between the saline- and ethanol-infusion groups, and these PCO$_2$ values are similar to those found in other studies conducted in our laboratory during the same time period.

In summary, maternal intravenous infusion of ethanol decreases fetal cerebral ECF PGE$_2$ concentration transiently and increases adenosine concentration in ovine fetuses under 125 days of gestation, in association with an inhibition of FBM, eye movements, and LV ECoG. Although these data do not unequivocally establish that adenosine is the mediator of ethanol-induced inhibition of FBM, they do demonstrate an important temporal relationship between increased fetal cerebral interstitial concentrations of adenosine and decreased FBM incidence during ethanol exposure. Overall, these data are consistent with adenosine, and not PGE$_2$, as the primary neuromodulator mediating the ethanol-
induced inhibition of FBM in the late-gestation ovine fetus under 125 days of gestation.

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