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RESEARCH REPORT

Neuroactive steroids induce changes in fetal sheep behavior during normoxic and asphyxic states

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Abstract
Allopregnanolone and related steroids are potent γ-aminobutyric acid receptor-A receptor agonistic allosteric modulators that suppress central nervous system (CNS) activity; in some species, these neurosteroids regulate normal CNS activity before birth. The aims of this study were to determine the effect of suppressing allopregnanolone production on behavioral responses to transient asphyxia in late gestation fetal sheep using the 5α-reductase (R)-2 inhibitor, finasteride. Specificity of the effects of finasteride was assessed by co-infusion of alfaxalone, a synthetic analog of allopregnanolone. Fetal catheters and electrodes for measurement of the electrocorticogram (ECoG) and nuchal electromyogram were implanted at 125 days of gestation, and an inflatable occluder was placed to allow umbilical cord occlusion (UCO). At ~ 130 days of gestation, fetuses received carotid arterial infusion of vehicle (2-hydroxypropyl-β-cyclodextrin; 40% w/vol), finasteride (40 mg/kg/h), alfaxalone (5 mg/kg/h), or finasteride + alfaxalone. A further three groups of fetuses were subjected to 5 min UCO at 30 min after the start of each infusion regime. Finasteride treatment alone increased the incidence of arousal-like activity; this was reduced by co-infusion of alfaxalone. After UCO, finasteride treatment caused a prolongation of sub-low voltage (LV) ECoG activity and increase in aberrant ECoG spike activity when compared to vehicle-treated UCO fetuses. After UCO, alfaxalone treatment reduced the incidence of sub-LV, reduced the number of aberrant EEG spikes, and restored ECoG activity to the pattern observed after UCO in vehicle-treated fetuses. These results confirm that neurosteroids significantly modulate normal CNS activity in the late gestation fetus, modify, and limit the effects of asphyxia on the brain.

Keywords: *Allopregnanolone, cortisol, finasteride, neuroprotection, sleep state, umbilical cord occlusion*

Introduction

Chronic or acute bouts of hypoxia/ischemia in late pregnancy can have serious consequences for the newborn, including long-term neurological impairment. In studies on fetal sheep, we have shown that the fetus is partially protected from the deleterious effects of *in utero* hypoxia by the placental production of progesterone and its conversion to neuroactive steroids in the fetal periphery and brain (Nguyen et al. 2004; Yawno et al. 2009). Endogenous steroids such as allopregnanolone act as allosteric modulators of the γ-aminobutyric acid receptor-A (GABA$_A$ receptor), prolonging GABA actions, and have an important role in regulating normal fetal behavior and central nervous system (CNS) activity (Nicol et al. 2001).

The great quantity of progesterone released into the fetal circulation in late gestation (Dolling and Seamark 1979) and the dramatic fall in levels of these steroids at birth (Nguyen et al. 2003), suggest that these steroids might be involved in maintaining fetal sleep, while their withdrawal at birth might allow arousal to occur. Progesterone is readily metabolized in the brain to 5α-dihydroprogesterone by the enzyme 5α-reductase type 2 (5αR-2), and then further reduced to allopregnanolone by the enzyme 3α-hydroxysteroid reductase. Allopregnanolone and other 3α-hydroxypregnanes interact with the steroid-binding site on the GABA$_A$ receptor to increase the effect of GABA on its receptor (Majewska 1992; Paul and Purdy 1992). From at least 0.7 days of gestation in fetal sheep, the GABA$_A$

receptor activity is inhibitory, and the effect of allopregnanolone on these receptors is therefore to suppress CNS activity (Nicol et al. 1998).

Assessment of fetal activity, specifically breathing movements and heart rate (HR) variability, is a key index for the monitoring of fetal health in human pregnancies (Nijhuis 2003). In fetal sheep, clear patterns of alternating episodes of high voltage (HV) and LV electrocorticogram (ECoG) activity are present from at least 120 days of gestation (term is ~ 147days; Clewlow et al. 1983). These ECoG patterns, together with other behavioral measures (e.g. eye movements and trunk muscle electromyogram (EMG)), are considered to be equivalent to "quiet" and "active" sleep episodes in adult animals and humans. These fetal "sleep" states are interrupted only briefly by short periods of arousal-like activity, characterized by LV ECoG activity coupled with the simultaneous appearance of postural muscle EMG activity, episodes of fetal breathing, and eye movements (Clewlow et al. 1983; Szeto 1992; Crossley et al. 1997; Nicol et al. 1998; Nicol et al. 2001). Under normal conditions, fetal arousal accounts for approximately 5% of total time, a low incidence attributed to the presence of GABA$_A$ receptor-active neurosteroids. Inhibition of either progesterone production or its conversion to 5α-reduced metabolites significantly increases the incidence of arousal-like activity in the sheep fetus (Crossley et al. 1997; Nicol et al. 2001).

In late gestation fetal sheep, acute global asphyxia rapidly induces cerebral expression of 5αR-2, resulting in increased allopregnanolone concentrations in extracellular fluid in the brain (Nguyen et al. 2004). This upregulation of allopregnanolone production appears to protect the brain since functional inhibition of 5αR-2 with finasteride increases the incidence of cell death in the fetal brain (Yawno et al. 2007). This is consistent with experiments in adult rats where stress-inducing paradigms have also been shown to increase 5αR-2 expression and allopregnanolone content in the brain (Purdy et al. 1991; Barbaccia et al. 1996). Thus, an important role of allopregnanolone may be to reduce the high level of neuronal excitation that follows acute episodes of fetal asphyxia during late gestation. Prolonged neuronal excitation may develop into episodes of seizure-like activity that can be observed during recovery from asphyxia in fetal sheep (Dean et al. 2006), activity that may further contribute to cell damage in the brain and which may be constrained somewhat by the presence of endogenous neurosteroids.

The aim of the current study was to investigate the effects of inhibiting 5αR-2 activity on normal fetal CNS activity, and then on the changes of activity that follow transient fetal asphyxia induced by brief episode of umbilical cord occlusion (UCO). We also examined the effect of infusing a synthetic analog of allopregnanolone, alfaxalone, on preventing asphyxia-induced changes in CNS activity. We hypothesized that

inhibiting 5αR-2 would alter fetal ECoG activities and increase the incidence of arousal-like behavior. Furthermore, we hypothesized that abnormal brain activity in the form of high amplitude spikes and seizure-like activity would appear after transient fetal asphyxia. Finally, we hypothesized that infusion of alfaxalone would decrease the effects of 5αR-2 inhibition and reduce the abnormal brain activity induced by asphyxia.

Methods

Animals and procedures

Thirty-four pregnant Merino-Border Leicester ewes purchased from an accredited private supplier in Meredith, Vic., Australia, of known gestational age carrying singleton or twin fetuses were used for this study. The use of these animals as well as the procedures performed had received prior approval from the Monash University Standing Committee on Ethics and Animal Experimentation. The animals were prepared for implantation of catheters as previously described (Yawno et al. 2007). Briefly, at 125 ± 2 days of gestation (term is *ca.* 147 days), surgery was performed under general inhalational anesthesia using isoflurane (Isoflo; Abbott, Australasia). Through a mid-line abdominal incision, the uterus was identified, and a 10–15 cm incision was made to exteriorize the fetus. A catheter was inserted into the right brachial artery of the fetus and advanced toward the heart until the tip of the catheter was located in the common carotid artery. As intravenous infusion would result in much of the finasteride being lost into the maternal circulation by crossing the placenta, this arterial catheter was used to infuse finasteride or vehicle so that the drug passed into the ascending carotid blood stream and through the cerebral circulation before entering the systemic circulation. A catheter placed in the left brachial artery was used to collect blood samples. An inflatable silastic cuff (In Vivo Medical, Ukiah, CA, USA) was placed around the umbilical cord which when inflated caused complete cessation of umbilical blood flow as described previously (Yawno et al. 2007). For the measurement of EcoG, two insulated stainless steel wire electrodes were inserted into 1 mm diameter holes made with a hand-held drill approximately 10 mm lateral to the sagittal suture and 5 mm forward of the coronal suture; i.e. overlying the left and right parietal cortex. A third electrode was sewn under the skin at the rear of the skull to act as the common electrode. Nuchal EMG was recorded from a pair of insulated stainless steel electrodes sewn into the dorsal muscles of the neck as previously described (Clewlow et al. 1983; Nicol et al. 1998). The fetus was then returned to the uterus, all incisions repaired, and the catheters exteriorized through a small incision on the right hand flank of the ewe. The ewe and fetus

were allowed 3–4 days of recovery before beginning the experiment.

Recordings analysis

Fetal mean arterial pressure (MAP) and HR were recorded throughout each experiment using pressure transducers and appropriate amplifiers, as described previously (Yawno et al. 2007). The fetal ECoG and EMG signals were recorded using a high common mode rejection amplifier (Wide Band AC Pre-Amplifier; Model 7P3B, Grass Instrument Co., Quincy, MA, USA) incorporating a low-pass filter (30 Hz), and then digitized (Power Lab, AD Instruments, Castle Hill, NSW, Australia) and recorded on a computer using Chart 5 software (AD Instruments). The MAP, HR, EcoG, and EMG activities were recorded in all fetuses for at least 24 h prior to any treatments or experimental intervention. The amplitude of each signal was averaged every 2 min over the 2-h period immediately preceding the start of experimental procedures to establish the baseline or control levels of each parameter. ECoG was divided into episodes of low and high amplitude activity based on inspection of the signal, and a threshold was determined for each recording that discriminated accurately between LV and HV activities. Because the asphyxial insult produced by UCO resulted in (i) very low amplitude ECoG (here designated as either near-isoelectric activity or "sub-LV" [<50% average LV ECoG amplitude] persisting for >5 min) and (ii) ECoG spiking activity (defined as spike amplitude >50% average HV ECoG amplitude), criteria were also set to distinguish episodes of these activities in the ECoG record. Similarly, the presence or absence of nuchal EMG activity was scored on the basis of the signal amplitude during this pre-treatment control period. The LV, HV, and EMG thresholds were then applied to the whole record obtained from each fetus, using a macro written for analysis by the chart program, from which it was possible to determine episodes of fetal quiet sleep, active sleep, and arousal as previously described (Nicol et al. 2001). Briefly, fetal arousal was determined by the simultaneous presence of LV ECoG and nuchal muscle EMG activity.

Experimental design

Finasteride (20 mg/kg/h, based on estimated fetal weight, Steraloids Inc, New York, USA), alfaxalone (5 mg/kg/h, 3α-hydroxypregnane-11, 20-dione; Alfaxan-CD, Jurox, Rutherford, Australia) in 2-hydroxypropyl-β-cyclodextrin (40% w/vol, Sigma-Aldrich, Sydney, Australia), or an equivalent volume of vehicle (2-hydroxypropyl-β-cyclodextrin) was infused into the fetus at a rate of 5 ml/h for 2 h via the right brachial artery catheter. Complete solubilization of finasteride in the 40% 2-hydroxypropyl-β-cyclodextrin solution was achieved by sonication. The dose of finasteride used has been shown to be sufficient to inhibit 5α-R enzyme activity in late gestation fetal sheep (Nicol et al. 2001), and has been shown to significantly suppress allopregnanolone concentration in the fetal brain (Yawno et al. 2009). The dose of alfaxalone used has previously been shown to reduce hypoxia-induced cell death in the sheep fetus (Yawno et al. 2009).

Twenty fetuses received either finasteride ($n = 5$), alfaxalone ($n = 5$), both finasteride and alfaxalone ($n = 5$), or vehicle ($n = 5$) for 2 h at the doses indicated above. These fetuses were not subjected to UCO (i.e. the umbilical cuff was inserted at surgery but not inflated subsequently). Because a previous study had shown that finasteride infusion had an effect on 5α-R activity for some time after the infusion had been completed (Yawno et al. 2007), when treating fetuses with both finasteride and alfaxalone, the alfaxalone infusion (5 mg/kg/h) was given for an additional 3 h to compensate for this prolonged effect of finasteride on the 5α-R enzyme. A further 14 fetuses were subjected to transient asphyxia (5 min) induced by UCO starting 30 min after the beginning of the finasteride ($n = 4$), alfaxalone ($n = 5$), or vehicle ($n = 5$) infusions.

Fetal arterial blood samples were taken before, during, and after the induction of UCO. Samples were used immediately for the measurement of pH, O_2 saturation (sO_2), partial pressure of O_2 (PO_2), partial pressure of CO_2 (PCO_2), base excess, glucose, and lactate using an ABL 510 blood gas analyzer (Radiometer, Copenhagen, Denmark). Fetal blood gas measurements were corrected to the fetal temperature of approximately 39°C. All fetuses were killed 24 h after the start of infusions by i.v. injection of pentobarbitone sodium (130 mg/kg; Lethabarb, Virbac Pty. Ltd., Peakhurst, NSW, Australia) to the ewe.

Cortisol radioimmunoassay

Cortisol was extracted from plasma with dichloromethane and measured by radioimmunoassay as described previously (Bocking et al. 1986). The intra- and inter-assay coefficients of variance were 9% ($n = 5$) and 10% ($n = 5$), respectively, and the sensitivity of the assay was 0.42 ± 0.02 ng/ml.

Statistical analyses

Data are expressed as mean ± SEM. Two-way repeated-measures ANOVA with treatment and time as the factors was used to analyze the differences in blood gases, pH, MAP, HR, plasma cortisol concentrations, and ECoG and EMG activities between treatment groups and across time. Where the ANOVA indicated significant interaction between treatment and time, a Bonferroni *post hoc* test was applied to identify the significant differences between the treatments at each sampling time. The change of ECoG and EMG muscle activities from the control and pre-treatment levels, averaged over 2 h epochs,

was evaluated by one-way repeated-measures ANOVA, with a *post hoc* Dunnett's test for significance. Significant was reported as $P < 0.05$.

Results

Effect of finasteride and neuroactive steroid replacement on ECoG activity

All fetuses were alive and appeared healthy at the end of the 24-h recording period. Following treatment with finasteride, one-way repeated-measures ANOVA revealed significant main effects of time [$F(12,48) = 3.671$, $P = 0.001$], as the incidence of HV ECoG activity was significantly decreased between 4 and 24 h after the start of infusion, while the incidence of LV ECoG activity was increased when compared with pre-infusion values (Figure 1, upper panel). The co-infusion of finasteride and alfaxalone did significantly increase LV ECoG activity and decrease HV ECoG activity during the 2-h period of infusion, but these changes did not persist; from 4 to 24 h after beginning the infusion, the incidence of LV and HV ECoG
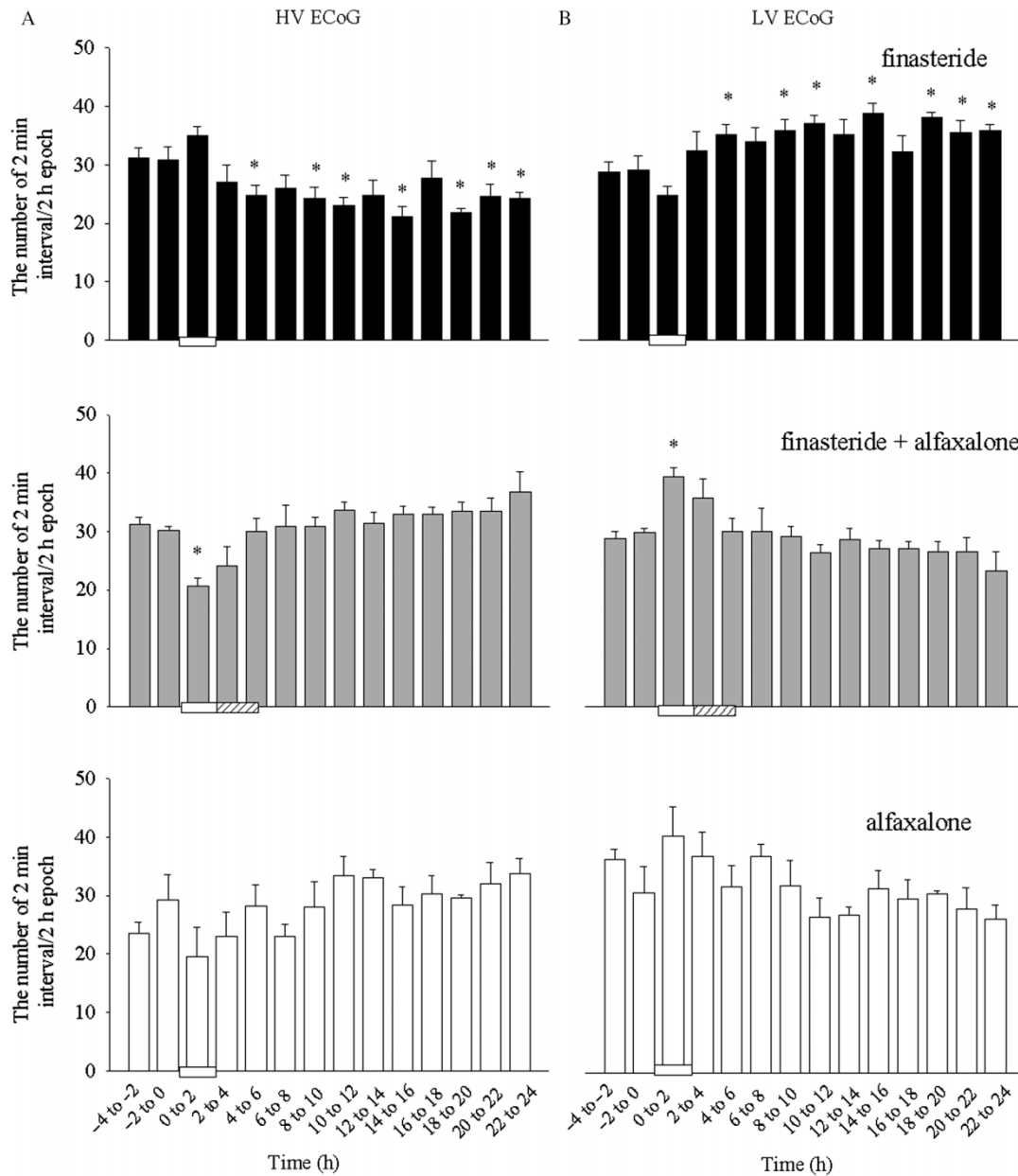

Figure 1. Effect of finasteride ($n = 5$; upper panels), finasteride + alfaxalone (middle panels; $n = 5$), and alfaxalone ($n = 5$; bottom panels) infusions via the fetal right brachial artery on the incidence of fetal (A) HV ECoG and (B) LV ECoG. Values are expressed as mean ± SEM of the incidence of each state per 2 h epoch, observed before, during, and after the start of infusions at time (0). The data were analyzed by two-way repeated-measures ANOVA, followed by Bonferroni post hoc group comparisons. The horizontal open bars on the X-axis indicate the time of the 2 h finasteride or finasteride + alfaxalone or alfaxalone infusion; the hatched bar in the middle panels indicates the extended 3 h alfaxalone infusion. *indicates significant difference compared with the pre-infusion values (-4-0 h pre-infusion period; $P < 0.05$). LV, low voltage; ECoG, electrocorticogram; HV, high voltage.

activities was not different from the pre-infusion period (Figure 1, middle panel). Alfaxalone infusion alone had no effect on ECoG activity compared to pre-infusion values (Figure 1, lower panel). For reasons of clarity, data from the untreated control groups are not shown in Figures 1–3, where no significant changes with time were present for any of the above variables over the 24 h of recording. While a diurnal rhythm for some variables (e.g. incidences of fetal breathing movements, HV and LV ECoG activities) might be expected to be present (Callea et al. 1990), in practice this is hard to distinguish and requires highly controlled environmental conditions to be expressed. We were unable to provide this sort of environment (in particular, controlled lighting and feeding conditions) and therefore have not made this type of analysis in this study.

Effect of finasteride and neuroactive steroid replacement on arousal-like activity

The incidence of fetal arousal (i.e. LV ECoG activity with concurrent nuchal EMG activity) was significantly increased in finasteride-treated fetuses compared to pre-infusion values (Figure 2, upper panel A). Two-way repeated-measures ANOVA revealed significant main effects of time [$F(13,117) = 4.4$, $P < 0.0001$] and experimental treatment [$F(2,9) = 17.36$, $P = 0.0008$]. In addition, the time by treatment interaction was also near significance [$F(26,117) = 1.57$, $P = 0.0544$]. The incidence of fetal arousal was highest at 14–16 h ($P = 0.01$) after starting the infusion. Finasteride treatment did not change the incidence of LV ECoG without nuchal EMG activity (i.e. activity normally defined as "active" or "rapid-eye movement" sleep; Figure 2, upper panel B). When alfaxalone was co-administered with finasteride, there was no change in the incidence of arousal-like behavior from pre-infusion values at any time after treatment (Figure 2, middle panel A). Alfaxalone treatment alone had no effect on arousal-like activity (Figure 2, lower panels).

Effect of finasteride and neuroactive steroid replacement on asphyxia–induced changes in fetal behavior

UCO for 5 min resulted in a marked decrease in the incidence of HV ECoG for up to 22 h after occlusion and a decrease in the incidence of LV ECoG for up to 6 h (Figure 3A and B). After UCO, very low amplitude (sub-LV) ECoG activity was observed that was not seen before the UCO (Figure 3C, upper panel). When the fetus was treated with finasteride, the incidence of sub-LV ECoG activity following UCO was further increased compared to the vehicle treatment, persisting for up to 6 h after the UCO (Figure 3C, middle panel). Two-way repeated-measures ANOVA revealed significant main effects of time [$F(13,143) = 5.347$, $P < 0.0001$] but not treatment [$F(2,11) = 2.42$, $P = 0.134$]. In addition, the time by treatment

interaction was also significant [$F(26,143) = 1.642$, $P = 0.036$]. In contrast, when the fetus was infused with alfaxalone, the incidence of sub-LV ECoG activity following UCO was significantly less than for the vehicle + UCO and finasteride + UCO fetuses (Figure 3C, lower panel). High amplitude spikes in ECoG occurred in all the fetuses after UCO (Figure 4). These spikes first appeared during the 5 min period of UCO and persisted for up to 25 min after UCO. As the spike amplitude was *ca.* 50% that of the average HV ECoG amplitude, it became harder to distinguish when sustained HV activity began to return to the ECoG pattern, which occurred at different times in different animals. Therefore, we selected the time frame of 25 min following UCO because during that time it was possible to unequivocally identify these spikes (Figure 5A). Finasteride treatment markedly increased the number of spikes between 15 and 20 min after UCO when compared with vehicle + UCO fetuses (Figure 5B), and infusion of alfaxalone resulted in a decrease in the ECoG spike activity between 10 and 25 min after UCO compared to vehicle-treated UCO fetuses (Figure 5C). In addition, alfaxalone infusion markedly reduced the incidence of ECoG spikes after UCO when compared to the finasteride + UCO fetuses.

Fetal physiological effects following UCO

UCO for 5 min significantly reduced fetal pH, O_2 saturation, PO_2, base excess, glucose, and HR, but these values returned to the normal range soon after release of the cuff. UCO increased PCO_2, lactate, and MAP significantly within 5 min, and these values also returned to the normal range after the release of the cuff ($P < 0.05$; Table I). Infusion of finasteride, alfaxalone, or vehicle had no effects on fetal blood gas parameters, or on the MAP and HR changes that occurred in response to UCO over the 24 h after occlusion (data not shown). Plasma cortisol concentrations were measured after UCO: the increase was similar in fetuses receiving vehicle or finasteride infusion. In contrast, infusion of alfaxalone effectively blocked the increase of cortisol concentration produced by UCO (Table II).

Discussion

The key findings of this study are that finasteride treatment increased arousal-like behavior in the sheep fetus and increased the incidence of sub-LV ECoG and abnormal spiking activity following severe global asphyxia produced by transient UCO. These activity patterns are indicative of an increased risk of brain injury; hyperexcitability and excitotoxicity after hypoxia have been major contributors to brain injury (Ben-Ari and Holmes 2006; Dean et al. 2006). The findings also support the concept that neuroactive steroids normally suppress CNS activities that
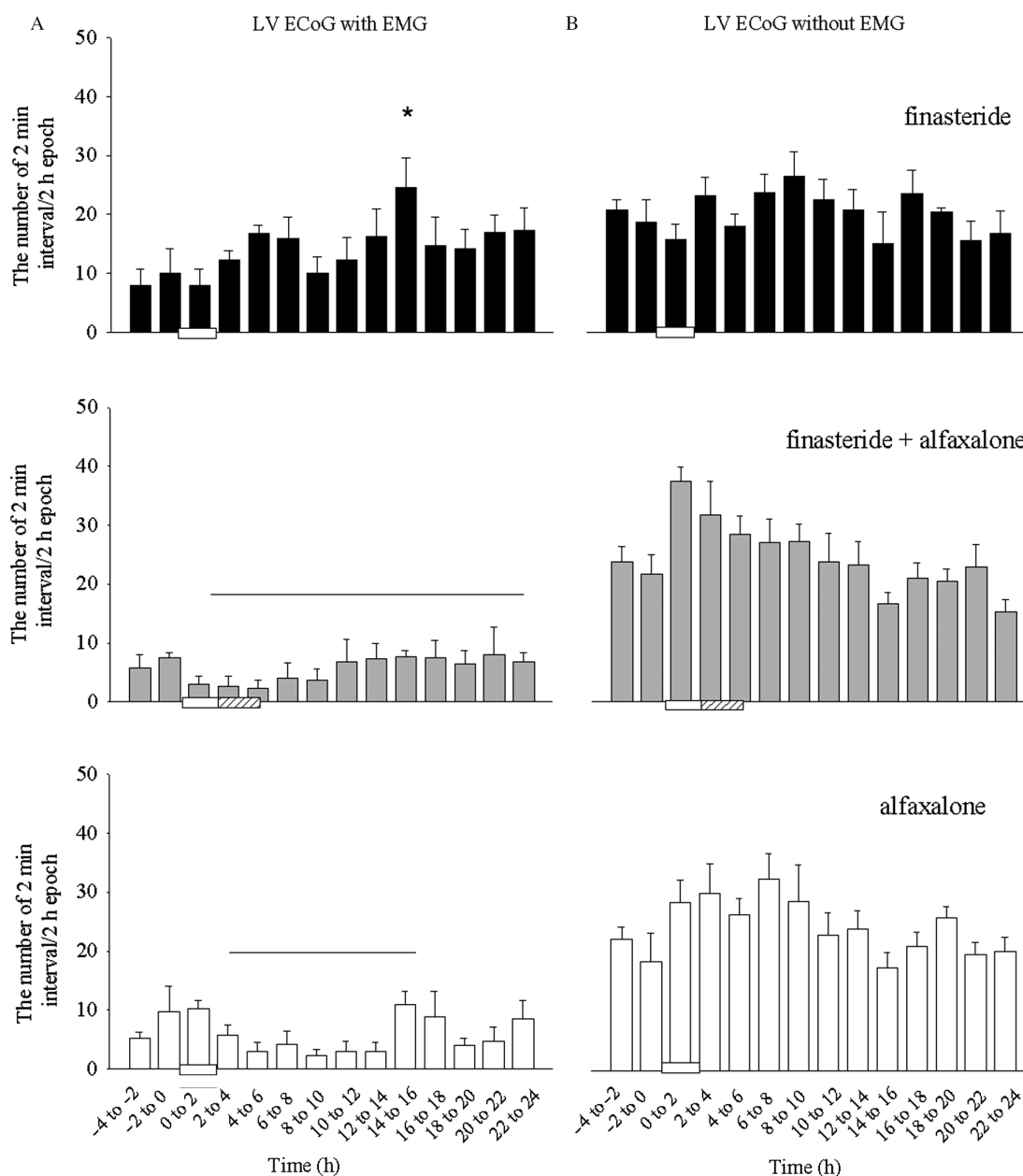
Figure 2.   Effect of finasteride ($n = 5$; upper panels), finasteride + alfaxalone ($n = 5$; middle panels), and alfaxalone ($n = 5$; bottom panels) infusions via the fetal right brachial artery on the incidence of the simultaneous presence of LV ECoG with nuchal EMG (A) and without nuchal EMG (B). Values shown are expressed as mean $\pm$ SEM of the incidence of each state per 2 h epoch. The data were analyzed by two-way repeated-measures ANOVA, followed by Bonferroni post hoc group comparisons. The horizontal open bars on the X-axis indicate the time of the infusions; the hatched bar in the middle panels indicates the extended alfaxalone infusion. The horizontal lines indicate significant differences compared to the finasteride + UCO group. *indicates significant difference from pre-treatment values. LV, low voltage; ECoG, electrocorticogram; EMG, electromyogram; UCO, umbilical cord occlusion.

contribute to hypoxia/ischemia-induced damage in the developing brain. In addition, the present study shows that the induction of spiking activity might be another pathway that contributes to the increased incidence of cell death seen in these fetuses (Yawno et al. 2007). This is further demonstrated by the finding that alfaxalone suppressed the changes in ECoG activity induced by asphyxia, and supports the hypothesis that the allopregnanolone analog would decrease the effects of 5αR-2 inhibition and reduce the abnormal brain activity induced by asphyxia.

The definition of arousal used in this study – the co-presence of LV ECoG activity and postural (in this case, nuchal) muscle EMG activity – has been used widely (De Vries et al. 1988; Szeto 1992; Nicol et al. 2001; Nijhuis 2003). The release of progesterone and some of its metabolites from the placenta into the fetal circulation has an important role in suppressing fetal arousal in late gestation, an effect requiring the conversion of progesterone to GABA$_A$ receptor agonist metabolites (Crossley et al. 1997; Nicol et al. 1998; Nicol et al. 1999; Nicol et al. 2001). The finding
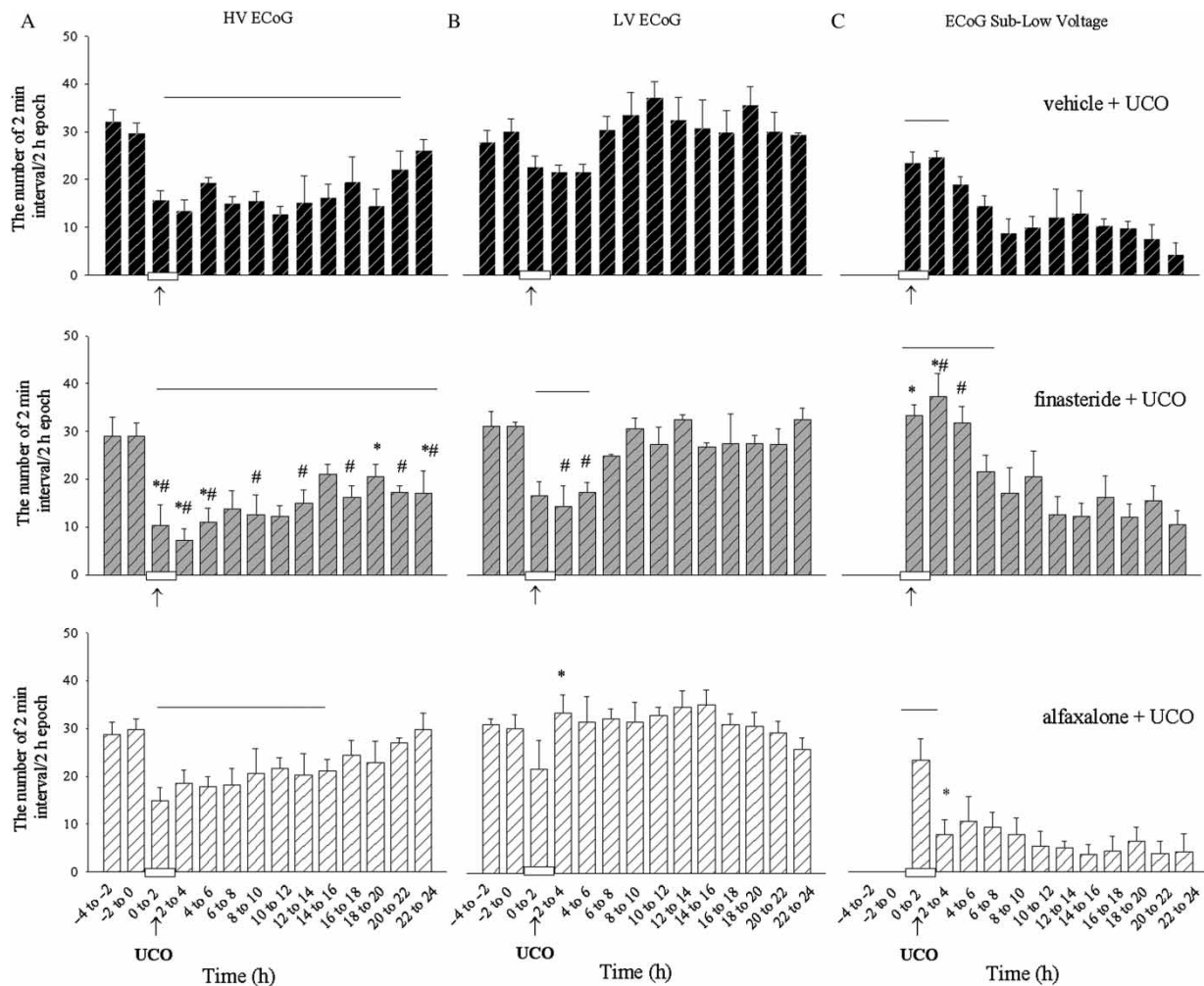
Figure 3.   Effect of UCO during vehicle (top panels; $n = 5$), finasteride (middle panels; $n = 4$), or alfaxalone (bottom panels; $n = 5$) infusions via the fetal right brachial artery on the incidence of HV ECoG (A), LV ECoG (B), and sub-LV activities (C). Values are expressed as mean ± SEM change in incidence of each state per 2 h epochs with respect to the start of infusion at time (0). The data were analyzed by two-way repeated-measures ANOVA, followed by Bonferroni post hoc group comparisons. The horizontal bars on the X-axis indicate the time of infusions (2 h). The arrows represent the UCO period that commenced 30 min after the start of infusion. The horizontal lines indicate significant differences between individual time points and pre-treatment values. The arrows represent the time of the 5 min UCO period commenced 30 min after the start of infusion. *indicates significant difference compared to vehicle + UCO group. #indicates significant difference between finasteride + UCO and alfaxalone + UCO group ($P < 0.05$). LV, low voltage; ECoG, electrocorticogram; HV, high voltage.

that finasteride, a potent inhibitor of the 5αR-2 enzyme (Guarna et al. 1998), altered the incidence of HV and LV ECoG from 4 to 24 h after its administration, and that fetal arousal-like activity was also increased, is consistent with previous studies on fetal behavior, in which finasteride was found to elevate the incidence of fetal arousal (Nicol et al.
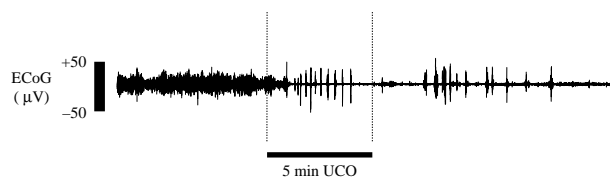

Figure 4.   ECoG recording from a sheep fetus that received 5 min UCO, showing the high amplitude spiking activity, during and after the 5 min UCO.

2001). The effect of finasteride on behavior in adult rats (Lephart et al. 1996; Frye and Bayon 1998) and fetal sheep (Nicol et al. 2001) is consistent with the inhibition of the metabolism of progesterone to allopregnanolone and the subsequent decrease over many hours of the availability of this steroid at the GABA_A receptor. It is, however, difficult to determine the potential contribution of other steroid pathways in the developing brain that may be inhibited by finasteride, and further studies are required to elucidate whether other 5α-reduced steroids act synergistically with allopregnanolone in the fetal brain.

The observation that alfaxalone suppresses CNS activity is consistent with the concept that allopregnanolone and related pregnane steroids have potent sedative effects on the fetus and maintain GABA_A receptor stimulation and the activity of GABAergic
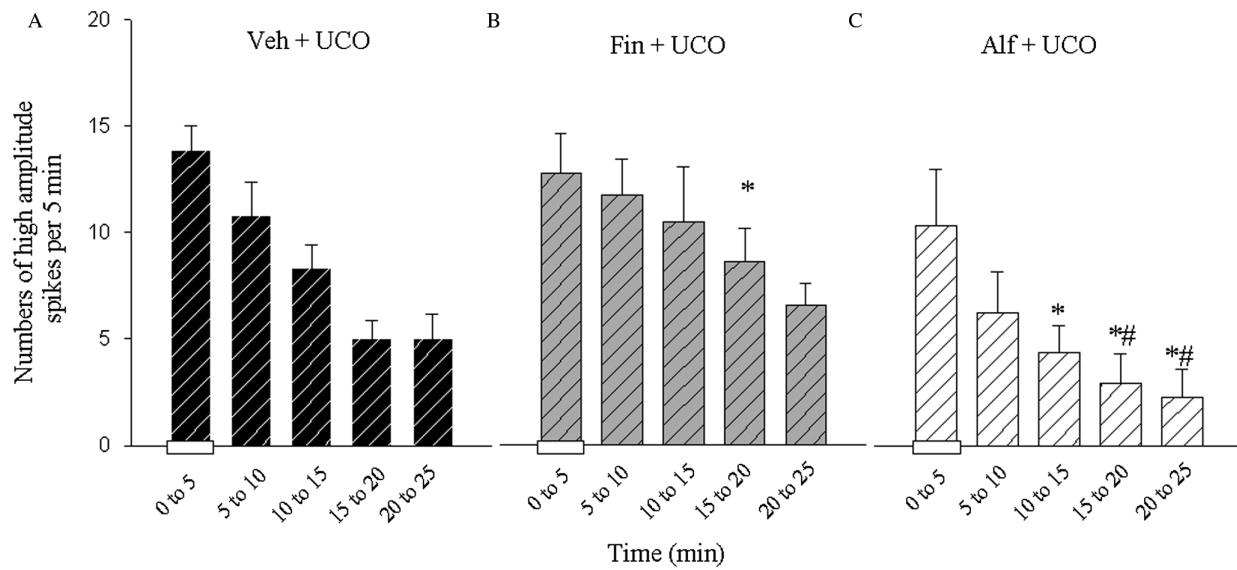
Figure 5.   The number of high amplitude spikes (number/5 min) in ECoG activity following UCO during vehicle (Veh, A; $n = 5$), finasteride (Fin, B; $n = 4$), or alfaxalone (Alf, C; $n = 5$) infusions via the fetal right brachial artery. The horizontal bars on the X-axis indicate the time of 5 min UCO. Values are expressed as mean $\pm$ SEM. *indicates significant difference from the vehicle + UCO group. #indicates significant difference between finasteride + UCO and alfaxalone + UCO groups ($P < 0.05$). ECoG, electrocorticogram.

Table I.   Arterial blood gases, pH, actual and standard base excess, glucose and lactate concentrations, MAP, and HR in fetuses that received vehicle + UCO or vehicle treatment alone.

		$-15\,min$	$+5\,min$	$+30\,min$	$+1\,h$	$+24\,h$
pH	Control	7.35 ± 0.009	7.36 ± 0.01	7.36 ± 0.01	7.36 ± 0.01	7.35 ± 0.01
	Veh + UCO	7.38 ± 0.02	7.04 ± 0.05★	7.28 ± 0.2	7.3 ± 0.02	7.35 ± 0.03
sO$_2$ (%)	Control	69.8 ± 5.9	70.1 ± 7.3★	69.1 ± 7	62.7 ± 4.8	67.6 ± 5.6
	Veh + UCO	68 ± 5.5	5.7 ± 0.6★	65 ± 4.9	68 ± 4.8	61 ± 9.3
PO$_2$ (mmHg)	Control	28.2 ± 5.4	23.5 ± 1.6	22 ± 2	22 ± 1.8	25.6 ± 1.8
	Veh + UCO	21.8 ± 1.5	4.2 ± 0.8★	23.5 ± 1.4	23 ± 1.3	20.4 ± 2
PCO$_2$ (mmHg)	Control	42.1 ± 2.2	41 ± 2.5	42 ± 2	40 ± 2.8	33.9 ± 4.9
	Veh + UCO	37.4 ± 1.48	93.1 ± 11★	41.4 ± 3.2	38.5 ± 2	40 ± 2
ABE (mmol/L)	Control	− 1.2 ± 0.7	− 3 ± 0.6	− 3 ± 1.4	− 2 ± 1.1	− 1.7 ± 0.8
	Veh + UCO	− 1.7 ± 0.4	− 9.2 ± 1.5★	− 6.7 ± 1.4★	− 4.9 ± 1★	− 4 ± 1.6★
SBE (mmol/L)	Control	− 1 ± 0.7	− 1.6 ± 0.3	− 2 ± 1.3	− 1.8 ± 1	− 1.7 ± 0.8
	Veh + UCO	− 1.9 ± 0.4	− 6.6 ± 1.3★	− 6.6 ± 1.5★	− 5 ± 1	− 4 ± 1.6
Glucose (mmol/L)	Control	0.6 ± 0.03	0.8 ± 0.06	0.9 ± 0.09	0.8 ± 0.1	0.7 ± 0.04
	Veh + UCO	0.6 ± 0.2	0.3 ± 0.1★	0.98 ± 0.3	0.8 ± 0.3	0.96 ± 0.05
Lactate (mmol/L)	Control	2.2 ± 0.8	2.1 ± 0.8	2.3 ± 0.6	1.8 ± 0.3	2.5 ± 0.1
	Veh + UCO	1.6 ± 0.2	5.7 ± 0.8★	4.7 ± 0.99	3.8 ± 0.9	0.96 ± 0.05
MAP (mmHg)	Control	41.4 ± 2.9	41.3 ± 4.1	40.8 ± 3.4	40.1 ± 1.1	42.2 ± 2.5
	Veh + UCO	33.7 ± 6.1	56.1 ± 6.7★	48.3 ± 2.08	43.5 ± 3.3	33.0 ± 4.7
HR (beats/min)	Control	161.4 ± 3.1	158.7 ± 2.2	156.9 ± 3.6	170.6 ± 5.4	161.4 ± 9.9
	Veh + UCO	147 ± 3.9	51 ± 9.2★	131.5 ± 9.1	122.3 ± 8.7	124.9 ± 13.5

Data are shown as mean $\pm$ SEM. $n = 5$ fetuses in each group. The data were analyzed by one-way repeated-measures ANOVA. ★$P < 0.05$, significant difference to pre-UCO values. ABE, SBE, actual and standard base excess; MAP, mean arterial pressure; HR, heart rate; UCO, umbilical cord occlusion; Veh, vehicle.

Table II.   Plasma cortisol concentration after UCO and during infusion (via the right brachial artery) of finasteride (Fin + UCO, $n = 5$), alfaxalone (Alf + UCO, $n = 5$), or vehicle (Veh + UCO, $n = 5$).

Treatment	$+1\,h$	$+2\,h$	$+5\,h$	$+24\,h$
Control	1.69 ± 0.59	1.16 ± 0.27	1.72 ± 0.56	1.08 ± 0.87
Veh + UCO	5.55 ± 1.39★	6.55 ± 1.74★#	5.68 ± 1.49★#	3.88 ± 1.1★
Fin + UCO	5.79 ± 2.37★	4.16 ± 0.56★#	5.49 ± 1.38★#	2.67 ± 0.7
Alf + UCO	3.35 ± 1.12	1.81 ± 0.24	1.87 ± 0.29	2.27 ± 0.59

Data are shown as mean $\pm$ SEM, and values are presented as fold increase from basal (pre-infusion). The data were analyzed by two-way repeated-measures ANOVA, followed by Bonferroni post hoc group comparisons. *indicates significant difference from control; # indicates significant difference from Alf + UCO group. $P < 0.05$.

inhibitory pathways in late gestation (Majewska 1992; Paul and Purdy 1992). Alfaxalone is a synthetic analog of allopregnanolone with a closely related structure, and it is a potent agonist at the steroid-binding site on the GABA$_A$ receptor (San Martin et al. 1996). We have recently reported that alfaxalone reduces the brain cell death induced by the suppression of endogenous neuroactive steroid levels, supporting a critical role for GABA$_A$ receptor agonist steroids in the developing brain (Yawno et al. 2007; Yawno et al. 2009).

In the current study, UCO alone caused a marked fall in oxygen saturation, pH, and changes in other physiological parameters in the fetal circulation, and although brief, these changes were serious enough to induce marked cell death (Yawno et al. 2007). The insult was also sufficient to cause a significant decrease in the incidence of HV ECoG and an increase in the incidence of sub-LV following UCO. The increase of the sub-LV ECoG occurred at a time (up to 4 h after UCO) when neurosteroid concentrations would be elevated in cerebral extracellular fluid (Nguyen et al. 2004). The finding that finasteride – (1) further decreased high and LV ECoG; (2) significantly prolonged the presence of the sub-LV compared to vehicle + UCO-treated fetuses; (3) induced ECoG high amplitude spiking activity, and (4) that alfaxalone prevented these changes – is further evidence of the important role that endogenous pregnane steroids such as allopregnanolone have in determining the outcome that adverse events (asphyxia, for example) have in the late gestation fetus. Other studies have shown that withdrawal of neuroactive steroids can lead to seizure-like activity in the adult rat (Frye and Bayon 1998; Kokate et al. 1999), and replacement with synthetic neuroactive steroids prevented these seizures (Reddy and Rogawski 2000; Rhodes and Frye 2005). Seizure-like activity following fetal asphyxia has been shown to contribute to cell death in the developing brain (Dean et al. 2006), and suppressing such asphyxia-induced changes in ECoG activity might be protective if brain damage can arise in this way.

Global asphyxia, produced by UCO, has been shown to induce an increase in the incidence of apoptotic and necrotic cell death in the brain of fetal sheep (Mallard et al. 1994; Castillo-Melendez et al. 2004). The fetal brain is able to respond to asphyxic insults with increased expression of P450scc and 5αR-2 in brain tissue, resulting in a rapid rise in allopregnanolone concentrations in the brain and cerebrospinal fluid (Nguyen et al. 2004). In the current study, neurosteroid actions were sustained for up to 24 h, which supports our earlier findings on the protective role of allopregnanolone after such a asphyxic/hypoxic episode (Yawno et al. 2007). It is likely that neurosteroids elicit actions more slowly than other inhibitory neurotransmitters, such as the purine adenosine (which is also likely to affect local cerebral blood flow by vasodilation), and that

neurosteroids provide a more global inhibition of neuronal activity. The finding that finasteride treatment markedly potentiated the effect of such episodes of asphyxia on ECoG activity again supports the concept that allopregnanolone has a key neuroprotective role in the fetal brain, possibly by combating the excitotoxicity that can result from excess release of excitatory neurotransmitters such as glutamate. The observation that alfaxalone reversed the effect of finasteride on ECoG activities, but alone had minimal effects on fetal behavior, is consistent with the concept that fetal activity is already markedly suppressed by the relatively high levels of endogenous neurosteroids normally present in the fetal brain.

The finding that fetal plasma cortisol concentrations were increased significantly from 1 to 5 h after UCO, during vehicle or finasteride administration compared to control fetuses, is consistent with the finding that an increase in plasma cortisol in fetal sheep is present following hypoxic (Billiards et al. 2006) or endotoxic (Billiards et al. 2002) events or following finasteride infusion (Yawno et al. 2009). This finding suggests that the hypothalamo-pituitary response to global fetal asphyxia might also include a neurosteroid-sensitive GABAergic pathway that regulates the release of ACTH and the adrenal stimulation that follows. For example, both cortisol and dexamethasone treatment increase glutamate release in the hippocampus (Iqbal et al. 2006). The effect appears to be partially related to the loss of allopregnanolone, since it was abolished by administration of alfaxalone.

In conclusion, suppression of neuroactive steroid synthesis with finasteride produced changes in behavioral parameters indicative of increased fetal arousal, and potentiated the abnormal ECoG patterns seen after UCO. Administration of alfaxalone reduced these responses, supporting the proposal that the synthesis of neuroactive pregnane steroids during late gestation and their modulation of the GABA$_A$ receptor contribute to the low levels of arousal activity present during fetal life. Alfaxalone treatment also reduced the potentially damaging pattern of ECoG spiking activity following UCO. We suggest that during pregnancy allopregnanolone strongly stimulates a specific population of GABA$_A$ receptors in the fetal brain that contain a delta subunit (Laurie et al. 1992) leading to the low level of arousal-like activity normally seen in the fetus. Therefore, any effect of alfaxalone supplementation will be relatively modest and not significant when administered alone, whereas a marked effect was seen when endogenous neuro-steroidogenesis was blocked with finasteride. As in humans, the half-life of alfaxalone in sheep is approximately 10 min; hence, alfaxalone would need to be infused after a hypoxic insult in clinical practice. We propose that this treatment would be most effective in reducing hyperexcitability when endogenous neurosteroid levels in the brain are reduced.

As neurosteroid levels fall at birth, supplementation with alfaxalone may be advantageous.

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