Prostaglandin D Synthase in the Prenatal Ovine Brain and Effects of Its Inhibition with Selenium Chloride on Fetal Sleep/Wake Activity *In Utero*

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It has been proposed that prostaglandin (PG) D_2 induces physiological sleep in mammals by acting on sleep centers located in the anterior hypothalamus. In fetal sheep, definitive rapid-eye-movement and non-rapid-eye-movement sleep states appear at ~125 d gestation (term is ~147 d). In adult animals, PGD synthase (PGDS) (functionally and structurally homologous to β-trace protein) is secreted into CSF with a circadian pattern, with the highest concentrations present during sleep. In this study we show that PGDS/β-trace protein is present in fetal sheep CSF at 125 and 135 d gestation but not at 90 d gestation. $SeCl_4$, a specific inhibitor of PGDS, was given to unanesthetized fetal sheep (130–140 d gestation) by intracerebroventricular infusion at a dose of 25, 100, 500, or 1000 pmol/min for 4 hr. Artificial CSF was infused in control experiments. Arousal behavior, defined as the presence of nuchal muscle electromyogram activity, electro-ocular activity, and breathing movements during low-amplitude electrocortical activity, increased from 3.8 ± 1 min/hr to 6.6 ± 0.5 and 7.0 ± 0.3 min/hr at doses of 100 and 500 pmol/min, respectively ($p < 0.05$). $SeCl_4$ at 25 and 1000 pmol/min had no significant effect on arousal activity. Infusion of PGD_2 at 500 pmol/min intracerebroventricularly for 4 hr decreased the incidence of arousal from 3.8 ± 0.5 min/hr to 0.7 ± 0.3 min/hr ($p < 0.05$). When 500 pmol/min PGD_2 was infused immediately after a 4 hr infusion of $SeCl_4$ (500 pmol/min), the $SeCl_4$-induced increase in arousal behavior was abolished. Together, the presence of PGDS/β-trace protein in fetal CSF in late gestation and the effects of $SeCl_4$ in increasing the incidence of arousal-like behavior suggest that PGD_2 has a role in the induction and maintenance of prenatal sleep.

Key words: fetus; sleep/wake; arousal; prostaglandin D synthase; β-trace protein; selenium chloride

In species with long gestations, considerable brain development occurs before birth; EEG and locomotor activities suggestive of sleep become evident by late gestation (Dawes et al., 1972; Ruckebusch et al., 1977; Clewlow et al., 1983; Szeto, 1992). In the sheep fetus, rapid-eye-movement (REM) and non-rapid-eye-movement (NREM) sleep, defined by the coordinated and regular changes in the electrocorticogram (ECoG), postural muscle electromyogram (EMG), electro-oculogram (EOG) activity, and episodes of breathing movements, are established by ~125 d (term ~147 d) gestation (Clewlow et al., 1983). These REM- and NREM-like episodes together account for ~95% of the total time (Dawes et al., 1972; Szeto and Hinman, 1985). The remaining time is occupied by brief periods identified as fetal arousal or wakefulness and characterized, as in the adult, by episodes of low-amplitude ECoG activity occurring simultaneously with EOG and nuchal muscle activities and augmented breathing movements (Szeto, 1992; Crossley et al., 1997; Nicol et al., 1998, 2001).

It is not known why the propensity to sleep is so high in fetal life. The placenta appears to have an influence over fetal CNS activity (Adamson et al., 1987), arising perhaps from the low-oxygen environment that results from the high oxidative metabolism of the placenta, or because somnogenic substances, especially neuroactive metabolites of progesterone, are released into fetal blood. Suprapontine control of fetal sleep was suggested by the observation that collicular transection disrupted the normal pattern of REM and NREM episodes (Dawes et al., 1983). However, little is known of the higher centers that might control the activity of the brainstem reticular network in the fetus and lead to the high incidence of sleep and low incidence of wakefulness *in utero*.

The anterior hypothalamus plays an essential role in sleep regulation (Sherin et al., 1996). It has been suggested that sleep induction occurs when prostaglandin (PG) D_2 activates GABAergic neurons in the ventrolateral preoptic (VLPO) region, which then inhibits histaminergic neurons in the posterior hypothalamus (Scammell et al., 1998). Extensive evidence suggests that PGD_2, formed from PGH_2 by the enzyme prostaglandin D synthase (PGDS), acts to induce physiological sleep (Hayaishi et al., 1993; Matsumura et al., 1994; Urade and Hayaishi, 1999; Hayaishi, 2000). PGDS has structural and functional homology to β-trace protein, a major constituent of CSF in humans and other species (Hoffman et al., 1993) with enzymatic properties similar to those of brain PGDS (Watanabe et al., 1994). PGDS/β-trace mRNA is found predominantly in choroid plexus and leptomeningeal cells (Urade et al., 1993, 1995; Hoffman et al., 1996; Ohe et al., 1996), and the protein is secreted into CSF. PGD_2 concentrations in CSF exhibit a circadian pattern (Pandley et al., 1995) and increase in CSF during sleep in the adult (Ram et al., 1997).

The question arises as to whether PGD_2 is an endogenous sleep substance in the fetus, as it is in the adult. This study was designed to investigate whether PGD_2 is involved in the maintenance of fetal sleep, first by determining whether PGDS was present in the

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CSF of fetal sheep and whether changes of content occurred in gestation around the time that sleep states first become evident; and second by making use of the observation that inorganic selenium compounds inhibit PGDS activity (Islam et al., 1991; Matsumura et al., 1991). $SeCl_4$ was infused into a lateral ventricle of fetal sheep *in utero* while recording sleep states, postural muscle activity, and breathing movements. Selenium compounds are specific and reversible inhibitors of brain-type PGDS because of their interaction with the cysteine-65 residue within the hydrophobic pocket of the enzyme, a structural feature of PGDS not shared with other members of the lipocalin superfamily (Nagata et al., 1991; Hayaishi, 2000). Infusion of $SeCl_4$ or PGD_2 alone and replacement of PGD_2 after $SeCl_4$ treatment established that PGD_2 induces sleep and that inhibition of PGDS with $SeCl_4$ induces increased amounts of an awake-like, aroused state in fetal sheep.

MATERIALS AND METHODS

Animals

Merino–Border Leicester crossbred ewes, which carry fetuses to 147 d gestation, were used in accordance with the rules of the Standing Committee on Ethics in Animal Experimentation of Monash University. The ewes were brought to the animal house and held together under artificially lit conditions on a 12 hr light/dark cycle for at least 5 d before surgery ($n = 13$) or before they were killed to collect the fetal brain ($n = 9$).

Ex vivo *studies: immunodetection of prostaglandin D synthase*

Fetal CSF and brain samples were collected at gestational ages of 90, 125, and 135 d ($n = 3$, each group). CSF was collected through a 23 gauge needle by puncturing the atlanto-occipital membrane immediately after the ewe had been killed and the fetus had been removed from the uterus. The fetal brain was then removed from the skull, the choroid plexus was removed from the lateral ventricles, and the remainder of the brain was divided into gross anatomical segments. The choroid plexus and brain samples were then frozen in liquid nitrogen. Samples of fetal liver and muscle were also collected for use as negative controls. At assay, the choroid plexus and selected segments of brain were weighed, pulverized on dry ice using an air hammer, and then homogenized in fresh buffer (in M: 0.1 phosphate, 0.32 sucrose, and 0.1 phenylmethylsulfonyl fluoride, pH 6) using an Ultra-Turrax (Janke and Kunkel GmbH, Staufen, Germany) homogenizer. The supernatant collected after centrifugation was subjected to SDS-PAGE electrophoresis on 12% acrylamide gel with a 4% stacking gel cast immediately before use. A lane containing protein standards between 14 and 97.4 kDa was included on each gel. Samples prepared from whole cortexes of adult rats ($n = 3$) and samples of human CSF collected by spinal lumbar puncture (gift from Dr. Samantha Richardson, University of Melbourne, Parkville, Australia) were used as positive controls. The proteins were transferred from the gel to a nitrocellulose membrane by electrophoresis at 4°C; complete transfer was confirmed subsequently by absence of Coomassie blue staining of the gel. The membrane was then treated with a protein blocking buffer (0.1 M phosphate, 0.05% Triton X-100, and 3% nonfat powered milk), and incubated for 1 hr with a 1:5000 dilution of a polyclonal antibody raised against β-trace protein purified from human CSF (gift from Professor M. Mader, University of Gottingen, Gottingen, Germany). After washing thoroughly in 0.1 M phosphate buffer containing 0.05% Triton X-100, the membrane was incubated for 1 hr in secondary antibody (goat anti-rabbit; 1:10,000) conjugated to horseradish peroxidase, and the bound antibody complex was visualized by enhanced chemiluminescence (ECL kit; Amersham Biosciences, Little Chalfont, UK) and exposure to film (Biomax Light; Eastman Kodak, Rochester, NY). The level of β-trace protein on the membrane was quantitated using the Image Tool program from the University of Texas Health Science Center (San Antonio, TX).

In utero *studies*

Surgery. The animals fasted for 24 hr before surgery at 125–126 d gestation for the long-term implantation of catheters and electrodes. The ewe was anesthetized using 1.5–2% halothane in oxygen for the implantation of maternal and fetal carotid artery and jugular vein catheters and the placement of catheters into the midcervical region of the fetal trachea and the amniotic sac. Pairs of multistranded stainless-steel wires (Cooner Wire Co., Chatsworth, CA) were sewn bilaterally into the nuchal muscles to measure EMG activity and subcutaneously at the margins of the orbit of one eye to measure the EOG (Clewlow et al., 1983). ECoG activity was recorded bilaterally from a pair of electrodes inserted through 1 mm diameter holes drilled through the skull over the parietal cortex. The bared ends of the insulated wires were inserted through the holes to rest on the dura, and the wires were then secured to the skull with cyanoacrylate glue. An indwelling cannula (inner diameter, 0.6 mm; Intracath; Terumo Medical Corporation, Tokyo, Japan) was then inserted into one lateral ventricle using a 22 gauge needle, as described previously (Hirst et al., 2000). After the needle and cannula had been inserted into the ventricle, the needle was withdrawn and the outer end of the Intracath was attached to a port in the base of a hollow cap machined from Delrin, which was then secured to the skull with cyanoacrylate adhesive. [The use of this device to infuse ultra-small volumes into the fetal ventricle is described more fully below and by Hirst et al. (2000).] The internal space of the cap and cannula assembly was filled with sterile artificial CSF (aCSF) by means of inflow and outflow catheters sealed into the wall of the cap. The fetus was then given a subcutaneous injection (2 ml) of a mixture of procaine penicillin (200 mg/ml) and dihydrostreptomycin (250 mg/ml) (Depomycin; Intervet Pty, Ltd., New South Wales, Australia). The uterus and membranes were repaired, and all catheters and wires were exteriorized from the abdomen through a 1 cm incision in the flank of the ewe. The abdominal incisions were then repaired and the ewe was allowed to recover. Experiments did not commence for at least 5 d after surgery.

Recordings. Tracheal pressure and carotid arterial pressures were recorded after electronic subtraction of amniotic pressure. Instantaneous heart rate was computed on-line from the pulse rate. The phasic changes of tracheal pressure were used as a measure of spontaneous fetal breathing movements. EOG and ECoG activities were amplified using wideband EEG pre-amplifiers (model 7P5B; Grass Instruments, West Warwick, RI) with frequency bandpass filters set at 1–15 Hz before being displayed directly on the polygraph. The EMG signal from the nuchal muscles was amplified, bandpass-filtered, and then integrated using a "leaky" integrator with the time constant set at 0.2 sec. All signals were recorded continuously for at least 24 hr before the experiments began, using a paper chart speed of 5 mm/min.

Experimental procedures. The internal volume of the catheters and cap attached to the intraventricular cannula was first filled with an aCSF suitable for fetal sheep (Bissonnette et al., 1981). The two catheters were attached to glass syringes mounted onto a double-barrel infusion pump (model 70134; B. Braun Melsungen AG, Melsungen, Germany); fluid was simultaneously infused and withdrawn through the catheters and cap. The greater internal diameter of these catheters (0.86 mm) compared with that of the intraventricular cannula (0.60 mm), and the use of this push/pull perfusion system, ensured that the fluid passed through the cap and catheters and did not enter the ventricle through the cannula. Sterility was maintained by attaching a 0.20 μm membrane filter (Minisart; Satorius AG, Gottingen, Germany) to the inflow syringe. Injection of known volumes of fluid into the ventricle was achieved by closing a stopcock on the outflow catheter for a known period of time, causing the fluid, which continued to be infused through the inflow catheter, to be forced through the cannula into the ventricle. Before commencement of infusion, both catheters and the cap were filled with the treatment solution. Infusion into the ventricle was done at a flow rate of 10 μl/min over 4 hr so that the total volume delivered over this time was 2.4 ml. $SeCl_4$ and PGD_2 were made up in aCSF. The infusion rate was constant and used for all treatments; the quantity of $SeCl_4$ or PGD_2 delivered into the ventricle (picomoles per minute) was varied by changing the concentration of each substance in the infusate. At the end of each treatment the catheters and caps were refilled with fresh aCSF, leaving ~10 μl of the treatment solution in the dead-space of the ventricular cannula.

Polygraph records of breathing movements, blood pressure, heart rate, ECoG, nuchal EMG, and EOG activities were obtained for at least 4 hr before and for 12 hr after commencement of each treatment. Fetal arterial blood samples (0.6 ml) were taken at hourly intervals for the measurement of blood gases, and the pH was corrected for the expected fetal body temperature of 38.5°C using an ABL5 (Radiometer, Copenhagen, Denmark) blood-gas analyzer. In the first part of the study, doses of $SeCl_4$ or PGD_2 between 25 pmol/min and 1 nmol/min were given to the fetuses to determine the effects on behavior. It was shown that 500 pmol of either $SeCl_4$ or PGD_2 produced maximal changes in fetal behavior, and in the final study, administration of 500 pmol/min of $SeCl_4$ for 4 hr was then followed immediately by a 4 hr infusion of 500

pmol/min of PGD_2 into the ventricle. To ensure that baseline conditions were achieved after each treatment, between 24 and 48 hr elapsed between treatments in each fetus.

Postmortem. Immediately before postmortem examination at 142 d gestation, the lateral ventricle was infused with 100 μl of Indian Ink (Winsor & Newton, London, UK), to verify correct insertion of the cannula into the lateral ventricle. The ewe was given an overdose of sodium pentobarbitone (325 mg/ml, i.v.; Lethobarb; Virbac, New South Wales, Australia) and the fetus was then immediately removed and weighed. In all the animals used for the study the cannula had passed through the cortex and the tip was observed to be in the lateral ventricle with no evidence of bleeding or tissue necrosis in the surrounding cortical tissue. Two fetuses in which the cannula had not entered the ventricle were excluded from the study.

Analysis of polygraph records. The entire 16 hr record of each experiment was analyzed on a minute-to-minute basis. For each minute, the ECoG, EMG, EOG, and fetal breathing movements were coded and then used to determine which behavioral state was present. NREM sleep was defined as high-amplitude ECoG activity (>100 μV) occurring simultaneously with sustained or tonic nuchal muscle EMG activity and the absence of EOG activity and breathing movements. REM sleep was considered to be present when the ECoG amplitude was of a low amplitude (<100 μV) in the presence of EOG activity and breathing movements and with no tonic nuchal EMG activity. As originally defined (Szeto and Hinman, 1985) and used previously (Crossley et al., 1997; Nicol et al., 1998, 2001), arousal was identified as periods of nuchal EMG, EOG, and breathing movement activities in the presence of low-amplitude ECoG. Arterial pressure and heart rate were measured from the record at 10 min intervals and then averaged over 1 or 4 hr epochs.

Statistical analysis

All data are presented as means ± SEM. All data were checked for homogeneity of variance using Levene's test of equality of error variances. If the data were not normally distributed, they were transformed by square root or natural log. Data were analyzed by repeated-measures ANOVA in which the between-subject factor was dose (0, 25, 100, 500, and 1000 pmol of $SeCl_4$ or PGD_2) and the repeated variable (within-subject factor) was time. When an interaction between factors was demonstrated, paired comparisons were made using the least significant difference test, and $p < 0.05$ was considered to be statistically significant.

RESULTS

Prostaglandin D synthase (β-trace protein) in CSF

Immunoreactive β-trace was detected in fetal CSF and adult human CSF (Fig. 1*A*) at an apparent molecular mass of 27 kDa, similar to that reported by others for this protein (Harrington et al., 1993). β-Trace protein was present in CSF taken from all fetuses at 125 and 135 d gestation but not in the samples from the three fetuses at 90 d gestation (Fig. 1*B,C*). There was no difference in expression of β-trace protein between fetuses at 125 and 135 d gestation. β-Trace protein was not detected in the fetal choroid plexus, liver, or skeletal muscle at any gestational age.

Intraventricular infusions of $SeCl_4$ and prostaglandin D_2

These experiments were performed in chronically catheterized and unanesthetized fetuses at 130–140 d gestation, *in utero*. In recordings made over 4 hr before infusion of either $SeCl_4$ or PGD_2, all fetuses showed normal behavioral states, in which clearly differentiated episodes of high- and low-voltage ECoG activities were present (Fig. 2*A*). The mean duration of the individual low- and high-voltage ECoG episodes was 7.7 ± 0.9 and 4.8 ± 0.8 min, respectively, and the average incidences for the control period were 33.4 ± 0.9 and 26.6 ± 0.9 min/hr. Of this, 25.1 ± 0.9 min/hr was occupied by the REM-like state (low-amplitude ECoG plus EOG activity and breathing movements) and 19.6 ± 1.0 min was occupied by the NREM-like state (high-amplitude ECoG plus nuchal EMG activity). Intermittent periods of activity, defined as arousal because of the presence of low-voltage ECoG activity together with nuchal EMG and EOG activities, occurred from time to time at the transition between the two sleep states (Fig. 2*A*). The mean duration of the individual arousal-like episodes was 1.5 ± 0.2 min; the mean incidence of arousal activity was 3.3 ± 0.3 min/hr. Arousal-like episodes were usually associated with a transient elevation of both blood

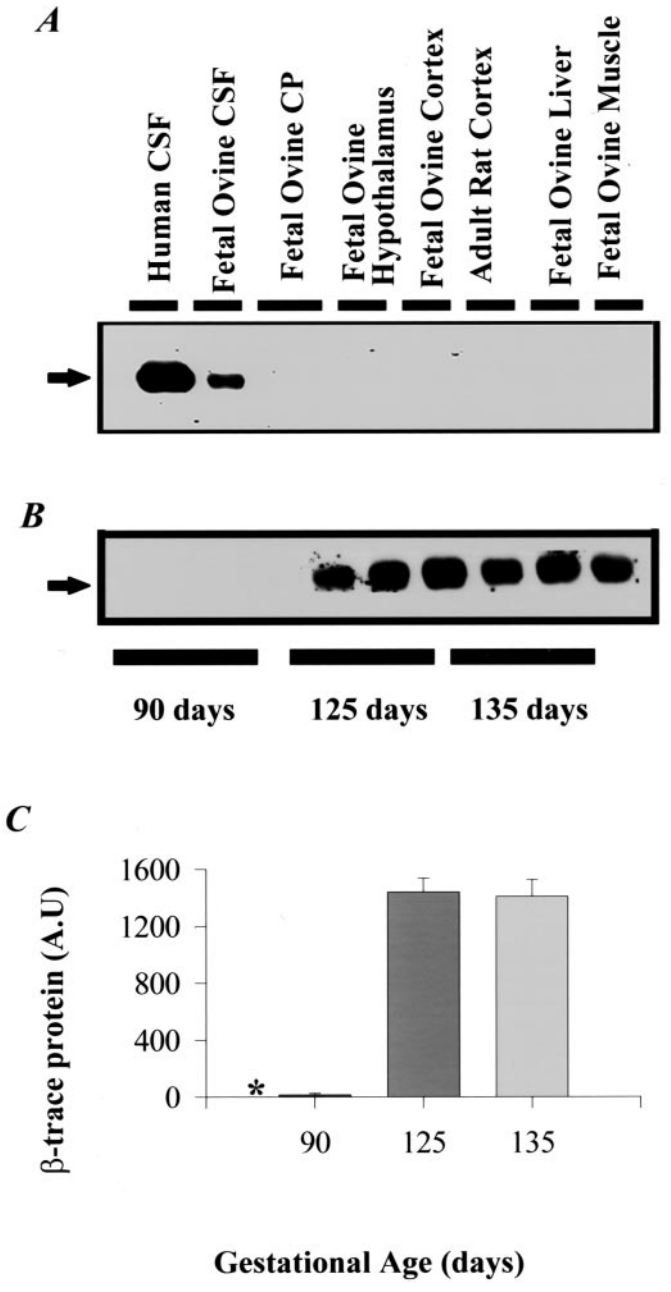

Figure 1. *A*, Western blot showing immunoreactive β-trace protein in adult human and fetal ovine CSF and its absence from fetal ovine choroid plexus (*CP*) hypothalamus, liver and muscle, and adult rat cortex. The molecular mass marker (27 kDa) shown by the *arrow* on the *left* was obtained from protein standards run on the same gel (data not shown). *B*, Western blot of immunoreactive β-trace protein in fetal ovine CSF at 90, 125, and 135 d gestation ($n = 3$ for each age). β-Trace protein was undetectable at 90 d gestation. The position of the 27 kDa molecular mass marker is shown by the *arrow* on the *left*. *C*, Densitometric analysis of β-trace protein expression is shown in *B*. Results shown are means ± SEM; the *asterisk* indicates a significant difference between the values at 90 d compared with those at 125 and 135 d gestation ($p < 0.05$).

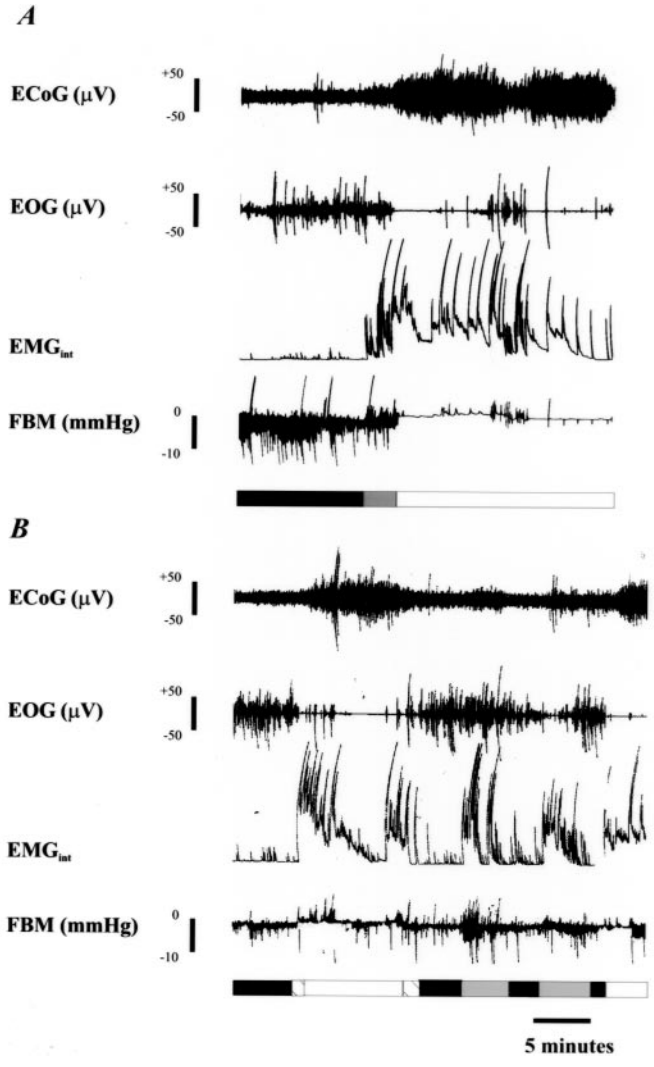

Figure 2. *A*, Polygraph recording during the pretreatment control period trace from a fetal sheep at 135 d gestation showing ECoG, EOG, integrated nuchal electromyogram (EMG_{int}) and fetal breathing movement (*FBM*) activities. REM sleep (*black bar*) was defined by the presence of FBM and EOG activity during low-amplitude ECoG activity. NREM sleep (*open bar*) was defined by the presence of varying but nearly continuous activity in the nuchal muscle during high-amplitude ECoG activity. Arousal (*shaded bar*) was identified by the presence of breathing movements, nuchal EMG, and EOG activities simultaneously with low-amplitude ECoG activity. *B*, Polygraph recording obtained from the same fetus shown in *A* >9 hr after the commencement of $SeCl_4$ at 500 pmol/min over 4 hr. Note the increase in the total amount of nuchal EMG and EOG activities and the increased incidence of the period defined as arousal (*shaded bars*). Periods that did not conform exactly to REM, NREM, or arousal behaviors are shown by the *hatched bars* at the beginning and end of the first NREM episode.

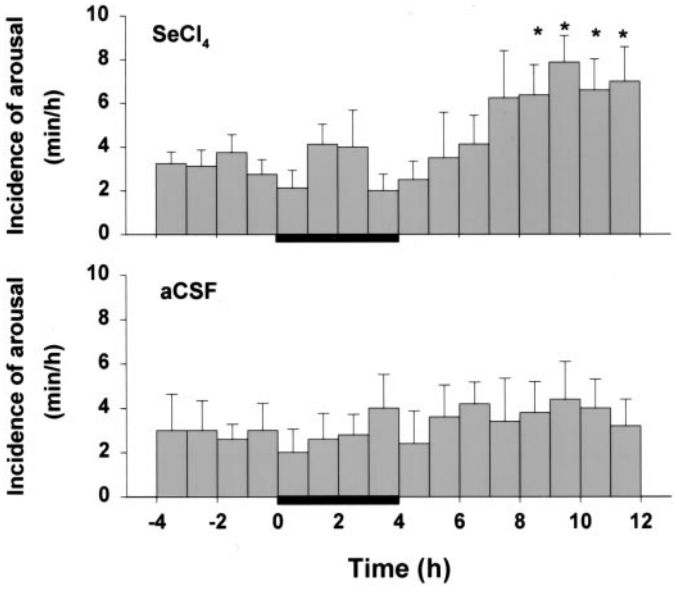

Figure 3. Effects of infusing aCSF ($n = 5$) or $SeCl_4$ at 500 pmol/min ($n = 4$) on the hourly incidence (minutes per hour) of arousal-like activity. Infusions were administered into the left ventricle of fetuses at 130–140 d gestation at 10 μl/min for 4 hr (*solid bar*). Administration of $SeCl_4$ caused a significant increase ($p < 0.05$, as shown by *asterisks*) in the incidence of arousal compared with equivalent times for infusion of aCSF. Data shown are means ± SEM.

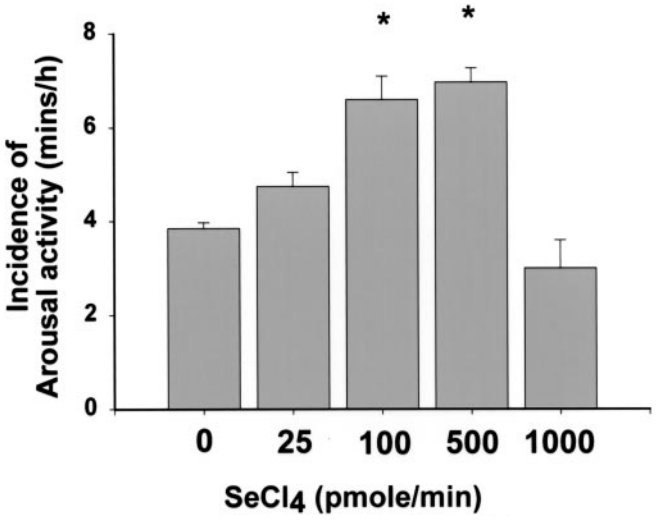

Figure 4. Effect of infusing $SeCl_4$ at 25, 100, 500, or 1000 pmol/min on the incidence (minutes per hour) of arousal behavior in fetal sheep. Zero dose was aCSF infused at 10 μl/min for 4 hr. Data shown are means ± SEM.

pressure and heart rate (data not shown). Because of either spurious artifact or record loss, ~6 min/hr could not be classified as REM, NREM, or arousal.

Infusion of $SeCl_4$ into the lateral ventricle of the fetus increased the number and overall incidence of arousal-like episodes (Fig. 2*B*). This effect was evident when a dose of 500 pmol/min was infused for 4 hr, resulting in a significant increase in arousal activity at 9, 10, 11, and 12 hr after starting the infusion (Fig. 3), compared with both the pretreatment values and the equivalent times during treatment with aCSF. Infusion of $SeCl_4$ at 25 pmol/min briefly increased arousal at 8–9 hr after the start of treatment but did not produce sustained effects, whereas infusions of either 100 or 500 pmol/min increased the arousal incidence from 3.8 ± 1 min/hr to 6.6 ± 0.5 and 7.0 ± 0.3 min/hr, respectively (Fig. 4). aCSF infused at 10 μl/min for 4 hr did not significantly alter any of the indexes of fetal behavior. Infusion of $SeCl_4$ at 1 nmol/min over 4 hr did not increase arousal activity (Fig. 4), but doses of 5 and 10 nmol/min produced seizure-like activity ($n = 4$; data not shown). At the doses below 1 nmol/min, $SeCl_4$ had no effect on the overall incidences of low- and high-voltage ECoG activities. When arousal-like activity was in-

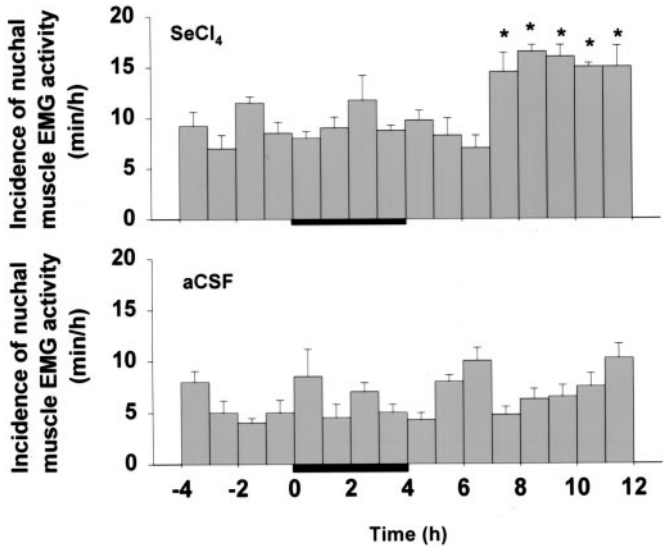

Figure 5. Effects of infusing aCSF ($n = 5$) or $SeCl_4$ at 500 pmol/min ($n = 4$) on the hourly incidence of nuchal EMG activity in the presence of low-amplitude ECoG. Infusions were administered into the left cerebral ventricle of fetuses at 130–140 d gestation for 4 hr (*solid bar*). Administration of $SeCl_4$ caused a significant increase ($p < 0.05$, as shown by *asterisks*) in the incidence of nuchal EMG activity in the presence of low-voltage activity compared with equivalent times for infusion of aCSF. Data shown are means ± SEM.

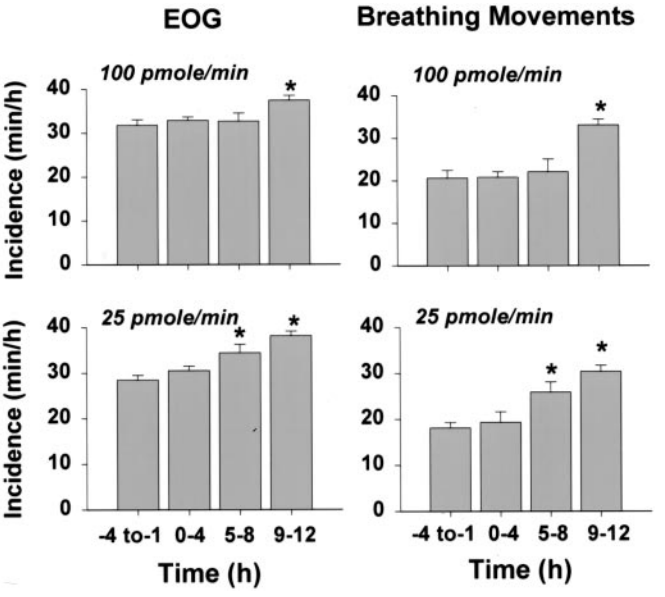

Figure 6. Effects of infusing $SeCl_4$ for 4 hr into the left cerebral ventricle of fetuses at 130–140 d gestation on the hourly incidence of EOG and breathing movements. Values were calculated by accumulating all activity over the 4 hr epochs and then determining the mean incidence per hour. Administration of $SeCl_4$ at 25 ($n = 4$) and 100 ($n = 4$) pmol/min caused a significant increase ($p < 0.05$, as shown by *asterisks*) in the incidence of nuchal EMG activity. Infusion of aCSF or the other doses of $SeCl_4$ had no effects on these parameters. Data shown are means ± SEM.

creased, this was attributable primarily to an increase in the incidence of nuchal muscle EMG activity that occurred when low-voltage ECoG activity was present (Fig. 5). At doses of 25 and 100 pmol/min, $SeCl_4$ also increased the incidences of EOG activity and breathing movements (Fig. 6).

The intraventricular infusion of PGD_2 alone had no significant effect on the incidences of low- and high-voltage ECoG activities, but the incidence of arousal was significantly decreased at doses of 500 and 1000 pmol/min. As shown in Figure 7, when PGD_2 was infused at 500 pmol/min the incidence of arousal-like behavior decreased from 3.8 ± 0.5 min/hr for the 4 hr control period to 0.7 min/hr at 6 hr after the start of the infusion. This decrease in activity began during the 4 hr infusion period, continued for 3 hr after the end of the infusion, and was attributable to decreased incidences of nuchal EMG activity and breathing movements during low-voltage ECoG (data not shown).

Four fetuses were then treated with $SeCl_4$ at 500 pmol/min for 4 hr, followed immediately by an intraventricular infusion of PGD_2 at 500 pmol/min for 4 hr. As described above, at 500 pmol/min $SeCl_4$ induced a significant increase in the incidence of arousal between 8 and 12 hr after starting the infusion attributable to the significant increase in nuchal EMG activity (Fig. 8). This increase in arousal was abolished by an infusion of PGD_2 at 500 pmol/min administered during the period 5–8 hr after the $SeCl_4$ treatment was started. The decline in the incidence of arousal was attributable to a reduction in the incidence of nuchal EMG activity during the epochs of low-amplitude ECoG activity (Fig. 8). The combined infusion of $SeCl_4$ and PGD_2 did not significantly alter the incidences of low- and high-amplitude ECoG activities, or when analyzed separately, the overall incidences of EOG activity or breathing movements.

Infusion of aCSF over 4 hr had no significant effect on the arousal-like behavior (Figs. 3 and 7) or nuchal muscle EMG activity exhibited by the fetuses (Fig. 5). Also, all separate and

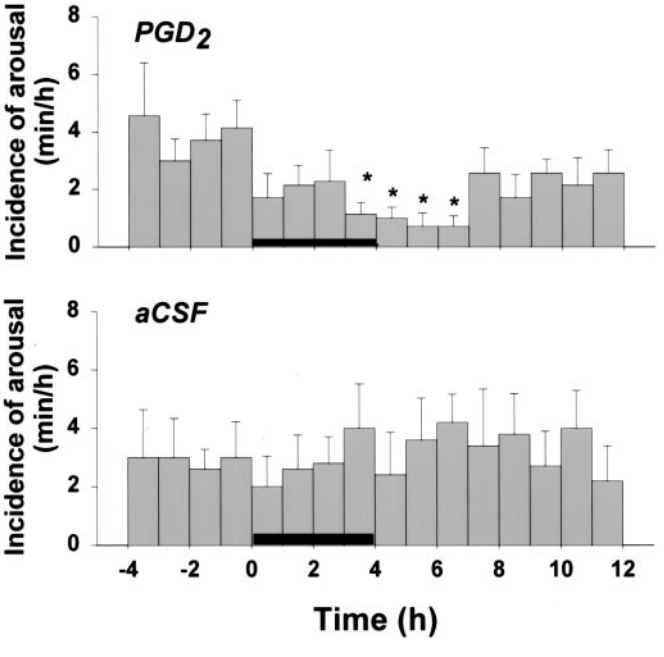

Figure 7. Effects of infusing aCSF ($n = 5$) or PGD_2 at 500 pmol/min ($n = 6$) on the hourly incidence (minutes per hour) of arousal. Infusions were administered into the left ventricle of fetuses at 130–140 d gestation at 10 μl/min for 4 hr (*solid bar*). PGD_2 caused a significant decrease in the incidence of arousal ($p < 0.05$, as shown by *asterisks*) compared with both the control period within the treatment group and with equivalent times for infusion of aCSF. Data shown are means ± SEM.

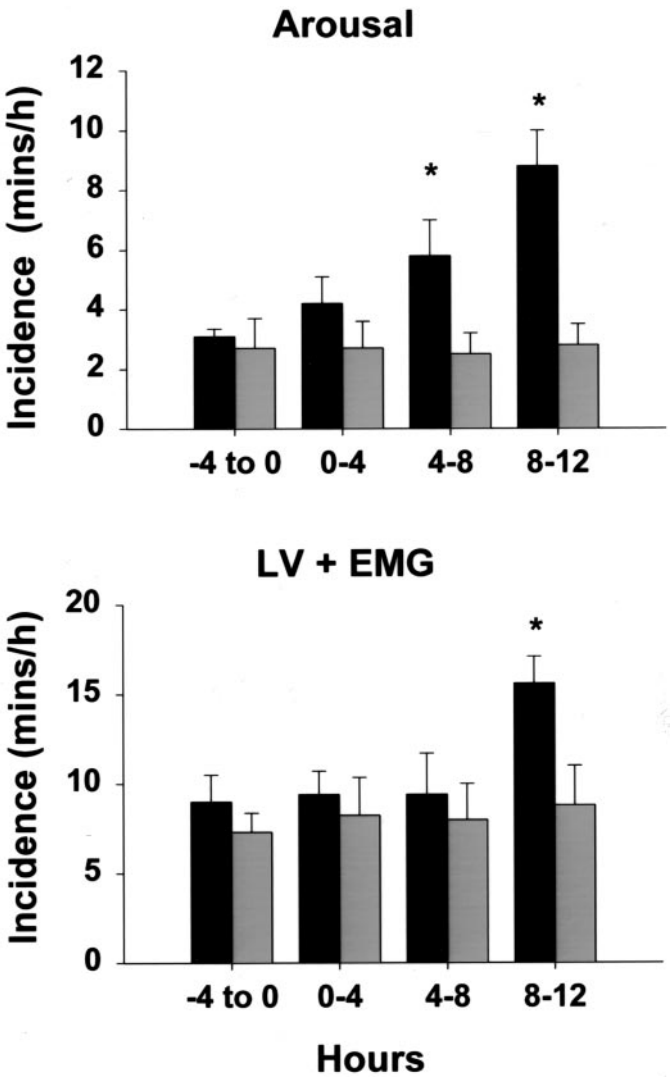

Figure 8. Effects of infusing $SeCl_4$ alone at 500 pmol/min (*solid bars*) or the same dose of $SeCl_4$ followed by a 4 hr infusion of PGD_2 at 500 pmol/min (*shaded bars*). The increased incidences of arousal and nuchal muscle EMG activities produced by the $SeCl_4$ treatments were abolished by the infusion of PGD_2. Data shown are means ± SEM ($n = 4$ fetuses) and demonstrate the incidence (minute per hour) of activity for consecutive 4 hr epochs.

combined treatments with $SeCl_4$ and PGD_2 did not have any significant effects on the arterial blood gases, pH, mean arterial pressure or heart rate (Tables 1 and 2). All of these parameters were in the normal range for fetal sheep at 130–140 d gestation.

DISCUSSION

We have shown that PGDS/β-trace protein was undetectable in the CSF of fetal sheep at 90 d gestation but was clearly present by 125 d gestation and thereafter. The appearance of this particular protein in CSF in late gestation is counter to the general trend for proteins to decrease in fetal CSF (Dziegielewska et al., 1980), although the CSF protein concentration at 125 d gestation (50 mg/ml) is still approximately twice that in adult sheep. The appearance of PGDS/β-trace protein in late gestation, at approximately the time that definite sleep states emerge, may be related to the onset of secretion of the protein from the choroid plexus and leptomeninges (Urade et al., 1995; Blodorn et al., 1996). The apparent absence of the protein from the fetal choroid plexus at any stage of development may be related to the low abundance of the protein in this tissue, because of its immediate secretion into CSF. Alternatively, the tertiary structure of the protein within the choroidal epithelial cells may differ from the secreted form. Similar results have been found in the human neonate, in which an antibody raised against the peptide sequence of human CSF β-trace did not recognize the protein in extracts of neonatal choroid plexus (Harrington et al., 1993).

An important finding of this study was that intraventricular infusion of $SeCl_4$ caused a dose-dependent increase in the incidence of fetal CNS activities, similar to that which defines arousal in the adult animal. This increase in fetal arousal was abolished by the subsequent administration of PGD_2 into the lateral ventricle. Infusion of aCSF at the same rate (10 μl/min) over 4 hr did not disrupt the ECoG, EOG, or nuchal muscle EMG activities, nor did it alter the patterns of activities from which the behavioral states of NREM and REM sleep and arousal are deduced. The production rate of CSF in late-gestation fetal sheep has been estimated to be 21.4 μl/min (Fossan et al., 1985) at ~125 d gestation, and the total CSF volume is likely to be turned over four to five times per day, as for most adult mammals (Davson et al., 1987). Thus, it is likely that the $SeCl_4$ would have been distributed throughout the CSF volume by the end of the 4 hr infusion period. The delayed onset of the increase in arousal-like behavior is thus consistent with the inhibition of an enzyme system and the subsequent slow decline of PGD_2 content in the brain and CSF. When PGD_2 was administered, the suppression of arousal began during the time of the infusion, suggesting that it had access to a site(s) that influences sleep and arousal activity in the brain. This is consistent with the lipophilic nature of PGD_2, which may enter the brain and reach the sites of action more rapidly compared with $SeCl_4$. For PGD_2, these sites are likely to be on the ventral surface of the rostral forebrain adjacent to the preoptic anterior hypothalamus (Matsumura et al., 1994; Scammell et al., 1998; Mizoguchi et al., 2001). The absence of any significant effects of the infusions on arterial pressure, heart rate, or blood gases also suggests that these treatments did not have significant nonspecific effects on brain functions.

The increase in the incidence of arousal behavior elicited by $SeCl_4$ occurred because of an increase in the both the total amount of nuchal muscle EMG activity and the amount of nuchal EMG, EOG, and breathing activities present during the low-amplitude ECoG state. At 100 pmol/min, $SeCl_4$ did not alter the total amount of nuchal EMG activity present, but there was a redistribution of the amount of nuchal muscle activity, with a greater incidence occurring during low-amplitude ECoG, when normally there is little sustained or tonic activity in this muscle group. At 500 pmol/min, $SeCl_4$ increased the total amount of EMG activity present in addition to an increase during low-amplitude ECoG activity.

The increase in arousal after $SeCl_4$ treatment implicates PGD_2 in the tonic regulation of sleep in the fetal sheep. The leptomeninges and choroid plexus contain abundant PGDS, but the PGD receptor is highly expressed in arachnoid trabecular cells in the vicinity of the basal forebrain/anterior preoptic region (Mizoguchi et al., 2001). PGD_2 appears to induce sleep by paracrine stimulation of adenosine release from these meningeal cells, which by exciting the adjacent sleep-active neurons in the VLPO leads in turn to GABAergic inhibition of the wake-promoting, histaminergic neurons in the tuberomammillary region of the posterior hypothalamus (for review, see Hayaishi, 2001). Block-

Table 1. Fetal arterial blood gases, pH, oxygen saturation, and hemoglobin concentration before (control) and at the end (+4 hr) of intracerebroventricular infusion of aCSF or $SeCl_4$ (500 pmol/min), or at +8 hr after $SeCl_4$ (500 pmol/min) and then PGD_2 (500 pmol/min)

	pH		P_{CO_2} (mmHg)		P_{O_2} (mmHg)		O_2 saturation (%)		Hemoglobin (%)	
Treatment	Control	+4 hr	Control	+4 hr	Control	+4 hr	Control	+4 hr	Control	+4 hr
aCSF	7.37 ± 0.010	7.38 ± 0.010	40.0 ± 3.0	39.7 ± 2.3	23.4 ± 2.3	25.3 ± 2.6	64.5 ± 6.9	67.9 ± 7.4	8.8 ± 0.5	8.8 ± 0.5
$SeCl_4$	7.40 ± 0.003	7.40 ± 0.003 +8 hr	42.2 ± 0.9	41.1 ± 2.7 +8 hr	26.4 ± 1.3	26.9 ± 0.9 +8 hr	73.9 ± 2.3	75.1 ± 2.5 +8 hr	10.5 ± 0.8	9.0 ± 1.2 +8 hr
$SeCl_4$ + PGD_2	7.37 ± 0.01	7.37 ± 0.01	40.3 ± 1.7	40.8 ± 1.5	23.3 ± 3.0	26.0 ± 1.4	70.5 ± 3.1	69.8 ± 1.1	9.8 ± 0.2	9.9 ± 0.3

Data shown are means ± SEM.

Table 2. Fetal arterial blood pressure and heart rate before (control) and at the end (+4 hr) of intracerebroventricular infusion of aCSF or $SeCl_4$ (500 pmol/min), or at +8 hr after $SeCl_4$ (500 pmol/min) and then PGD_2 (500 pmol/min)

	Mean arterial pressure (mmHg)		Heart rate (beats/min)	
Treatment	Control	+4 hr	Control	+4 hr
aCSF	48.2 ± 7.5	46.9 ± 5.3	163 ± 4.8	175 ± 11.9
$SeCl_4$	43.9 ± 5.1	46.3 ± 4.9 +8 hr	175 ± 12.8	174 ± 7.0 +8 hr
$SeCl_4$ + PGD_2	39.9 ± 6.1	38.2 ± 6.0	157 ± 7.5	158 ± 2.4

Data shown are means ± SEM.

ade of adenosine type $2A_2$ receptors in fetal sheep leads to increased high-voltage ECoG activity and NREM-like sleep in fetal sheep (Koos et al., 2001). In addition, we have shown that neurosteroid modulation of $GABA_A$ receptor activity leads to alterations of the fetal sleep states and arousal-like behavior (Crossley et al., 1997; Nicol et al., 1998, 2001). Thus, the neurotransmitters and neuromodulators of the putative hypothalamic sleep/wake system appear to have developed in the sheep brain by late gestation.

Another reason for the effect of $SeCl_4$ on fetal behavior might be that reduction of PGDS activity permitted increased production of PGE_2 from the common arachidonate precursor PGH_2. In adult rats it has been proposed that PGE_2 acts directly on posterior hypothalamic neurons to induce wakefulness (Matsumura et al., 1988). However, it should be noted that systemic infusions of PGE_2 or the PG synthase inhibitors indomethacin or meclofenamate do not alter sleep states or arousal in fetal sheep (Kitterman, 1987). It is possible that changes in the ratio of PGD_2 and PGE_2 concentrations in the fetal brain determine the propensity for sleep and wakefulness.

Whether the increases in nuchal EMG, EOG, and breathing activities during low-amplitude ECoG represent true arousal in the fetus must also be considered. Consistent with previous studies, transient increases in fetal blood pressure and heart rate have been observed during episodes identified as arousal (Szeto, 1992), and similar cardiovascular and autonomic changes occur in the newborn infant on arousal from sleep (Read et al., 1998). In a recent study we showed that evoked somatosensory responses were increased in amplitude after treating fetal sheep with the 5α-reductase inhibitor finasteride, a treatment that also increased the incidence of fetal arousal (Nicol et al., 2001). These observations are consistent with the episodes identified as fetal arousal as also being periods during which there is increased cortical excitability. Nevertheless, it is also of interest that the arousal-like episodes are brief, even after intracerebroventricular infusion of $SeCl_4$, suggesting that powerful sleep-promoting mechanisms remain active in the fetal sheep until the time of birth. The results of this study suggest that a PGD_2 mechanism contributes to the suppression of wakefulness in the fetus, but it is not the sole mechanism that maintains the sleep that accounts for most of the behavioral state in fetal life.

Other proposed functions of PGDS/β-trace protein may be important. It has been identified as a member of the lipocalin superfamily in the basis of conserved tertiary structure (Nagata et al., 1991), making it apparent that this protein may be involved in the transmembrane transport of small lipophilic molecules. In adults, β-trace protein is constitutively expressed at other blood–tissue boundaries, including the retina (Beuckmann et al., 1996; Gerashchenko et al., 1998) and testis (Tokugawa et al., 1998). Thus, this lipocalin-like protein may be involved in the transport of essential substrates into tissues that possess a microvasculature with barrier properties.

In summary, we have shown that inhibition of PGDS by $SeCl_4$ markedly increases the incidence of arousal-like behavior in late-gestation fetal sheep. This arousal-like activity was suppressed by PGD_2; PGD_2 replacement also prevented the actions of $SeCl_4$. These findings suggest that the activity of PGDS and production of PGD_2 in the fetal brain has a role in suppressing wakefulness and maintaining sleep *in utero*.

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