Effect of Dexamethasone Treatment on Maturational Changes in the NMDA Receptor in Sheep Brain

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The objective of the present study was to examine the effect of antenatal or postnatal treatment with corticosteroids on the NMDA receptor, one of the mediators of both normal brain development and hypoxic–ischemic injury, by determining the characteristics of the receptor MK-801 binding site in untreated and corticosteroid-treated fetal and newborn lambs. ^{3}H-MK-801 binding was performed in cerebral cortical cell membranes from fetal sheep at 88, 120, and 136 d gestation (term = 150 d), and from 5-d-old lambs and adult ewes. Animals were randomized to receive dexamethasone [fetuses: 6 mg, i.m. every 12 hr for four doses to mother; lambs: 0.01 mg/kg (low dose) or 0.25 mg/kg (high dose) every 12 hr for four doses] or placebo. During development, B_{max} (apparent number of receptors) increased, reaching a maximum in 5-d-old lambs ($p < 0.05$) and decreasing in the adult brain. K_d (dissociation constant) did not change, suggesting that receptor affinity was not altered during maturation. Dexamethasone treatment had no effect on MK-801 binding in the fetus or adult, but in lambs was associated with a significant decrease in B_{max} from 2.17 ± 0.18 pmol/mg protein in placebo-treated animals to 1.65 ± 0.8 and 1.62 ± 0.07 pmol/mg protein in low-dose and high-dose animals, respectively. Affinity for ^{3}H-MK-801 decreased 20% after dexamethasone treatment in lambs only ($p < 0.05$). Thus, dexamethasone treatment appears to modify the NMDA receptor only during a specific period of brain development.

Key words: NMDA; brain development; corticosteroids; glutamate receptors; receptor binding; sheep

Antenatal administration of corticosteroids has been shown to enhance maturation of several fetal organ systems. Maternal treatment with corticosteroids decreases the incidence of respiratory distress syndrome caused by surfactant deficiency in preterm infants by accelerating maturation of Type II alveolar cells and increasing surfactant production (Crowley et al., 1990; National Institutes of Health Consensus Development Panel, 1995). In addition to its effects on the lungs, fetal exposure to corticosteroids enhances renal and cardiovascular function and skin maturation (Stonestreet et al., 1983; Berry et al., 1997; Derks et al., 1997; Morikawa et al., 1998). However, antenatal corticosteroid administration may have both beneficial and detrimental effects on immature brain. The incidence of intraventricular hemorrhage is decreased in preterm infants exposed to corticosteroids *in utero*, and such treatment may decrease the incidence of long-term neurodevelopmental abnormalities (Garland et al., 1995; Ment et al., 1995). However, exposure to antenatal corticosteroids has also been associated with decreased head circumference and changes in visual memory (MacArthur et al., 1982; French et al., 1999). Furthermore, studies in animal models have demonstrated alterations in hippocampal volume (Uno et al., 1994), degeneration of hippocampal neurons (Uno et al., 1990), and delayed myelination after exposure to corticosteroids *in utero*.

The NMDA receptor is an ionotropic glutamate receptor found in the CNS with several well characterized regulatory and functional binding sites, including the glutamate (recognition) site, the glycine (co-activator) site, and a site within the receptor-associated ion channel that binds phencyclidine and the noncompetitive antagonist MK-801 (dizocilpine) (Wood et al., 1990). The number and distribution of cerebral NMDA receptors changes during fetal and neonatal development, reflecting the role played by NMDA receptor activity in modulating long-term potentiation and synaptogenesis, both active processes in immature brain (Cotman et al., 1994; Asztély and Gustafsson, 1996). However, excessive activation of the NMDA receptor may lead to excitotoxic brain injury (Rothman and Olney, 1986; Choi, 1990; Hagberg et al., 1992). We hypothesized that steroid treatment might alter the maturational changes in the NMDA receptor during gestation, thus contributing to the observed effects of corticosteroid treatment on immature brain. The objective of the present study was to examine the effect of antenatal and postnatal treatment with corticosteroids on the cerebral NMDA receptor during brain development by determining the characteristics of the ion-channel MK-801 binding site in untreated and treated fetal and newborn lambs.

MATERIALS AND METHODS

Experimental design. Studies were performed in pregnant ewes at 88, 120, and 136 d gestation (term = 150 d) and 5-d-old lambs. All animal protocols were approved by the Institutional Animal Care and Use Committees of Brown University and Women and Infants' Hospital of Rhode Island.

Animal preparation. Cerebral cortical tissue used in this study was obtained from animals being studied under a second protocol to determine the effects of antenatal and postnatal corticosteroids on the blood–brain barrier. Therefore, catheters were placed in accordance with the second protocol (Stonestreet et al., 1999), which did not use any agents known to have an effect on the ovine NMDA receptor. In the prenatal treatment group, surgery was performed using 1–2% halothane for anesthesia in pregnant ewes at either 84, 112–113, or 128–130 d gestation, as previously described (Stonestreet et al., 1983, 1993). Fetal catheters were placed in a brachial vein and in the thoracic aorta via a brachial artery. An amniotic fluid catheter and a maternal femoral artery catheter were also placed. After a 4–7 d recovery period, ewes at each gestational age were randomly assigned to receive either dexamethasone (Fujsawa USA, Deerfield, IL) (6 mg, i.m. every 12 hr for a total of four doses), or an equal volume of placebo (0.9% NaCl w/v in H_2O). This dosing regimen is similar to that used in pregnant women to enhance fetal lung maturation and was selected to maximize corticosteroid effects while minimizing the risk of premature labor in the ewes (Derks et al., 1997). Dexamethasone was selected because either dexamethasone or its stereoisomer, betamethasone, are the corticosteroids of choice for antenatal therapy to accelerate fetal maturation as well as postnatal therapy in newborns with respiratory distress syndrome or bronchopulmonary dysplasia.

Received Nov. 12, 1999; revised May 9, 2000; accepted July 19, 2000.

This work was supported by National Institutes of Health Grants HD20337 and HD 34618.

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Table 1. Physiologic data from fetal and newborn lambs

		88 d	120 d	136 d	5-d-old lambs
pH	Placebo	7.37 ± 0.01 (3)*	7.33 ± 0.01 (4)	7.31 ± 0.03 (4)	7.40 ± 0.01 (5)
	Dexamethasone	—	7.33 ± 0.02 (3)	7.38 ± 0.03 (5)	LD, 7.38 ± 0.01 (6) HD, 7.42 ± 0.02 (6)
$PaCO_2$ (mmHg)	Placebo	53 ± 1	49 ± 5	51 ± 1	37 ± 1
	Dexamethasone	—	43 ± 2	48 ± 2	LD, 44 ± 3** HD, 36 ± 2
PaO_2 (mmHg)	Placebo	28 ± 1	26 ± 1	21 ± 2	97 ± 6
	Dexamethasone	—	30 ± 4	21 ± 2	LD, 88 ± 4** HD, 104 ± 10
Heart rate (bpm)	Placebo	197 ± 33	174 ± 18	164 ± 6	244 ± 8
	Dexamethasone	—	213 ± 22	185 ± 9	LD, 215 ± 25** HD, 254 ± 30
MABP (mmHg)	Placebo	33 ± 10	53 ± 14	59 ± 8	54 ± 4
	Dexamethasone	—	61 ± 11	55 ± 5	LD, 52 ± 4** HD, 54 ± 8
Blood glucose (mg/dl)	Placebo	33 ± 2	19 ± 4	20 ± 7	139 ± 6
	Dexamethasone	—	36 ± 2	34 ± 10	LD, 118 ± 11** HD, 159 ± 10

*Mean ± SD (*n*).

**$p < 0.05$ vs placebo and HD (HD, high dose; LD, low dose).

To determine the effect of postnatal corticosteroid treatment, arterial and venous catheters were placed via the femoral vessels in 2-d-old lambs using 0.5–1% halothane for anesthesia. After a 24 hr recovery period, the lambs were assigned to receive either low-dose dexamethasone (0.01 mg/kg, i.m. every 12 hr for a total of four doses), high-dose dexamethasone (0.25 mg/kg every 12 hr for a total of four doses), or an equal volume of placebo. Low and high doses were selected to match the level of fetal exposure during prenatal steroid treatment and doses used for postnatal steroid treatment of bronchopulmonary dysplasia, respectively.

Fetal and newborn cerebral cortical tissue was obtained 12–18 hr after the fourth dose of corticosteroids was administered, frozen rapidly in liquid nitrogen, and stored at −80°C for further analysis. Cerebral cortex from mothers of study fetuses was also obtained after fetal tissue was harvested and similarly frozen and stored. Arterial blood gases, heart rate, and mean arterial blood pressure were determined at 50, 30, and 0 min before cerebral cortex was obtained. Blood samples for determination of serum glucose and cortisol levels were drawn via the arterial catheter at the time that tissue samples were obtained. Serum glucose concentrations were measured using a YSI 2300 STAT dual channel analyzer (YSI, Yellow Springs, OH). Serum cortisol levels were determined by an ^{125}I-radioimmunoassay method using a commercially available kit (Incstar, Stillwater, MN). The coefficients of variation for intra-assay and inter-assay precision for the cortisol assay were 10.1 and 7.9%, respectively.

Tissue analysis. Membranes were prepared by a modification of the method of Williams et al. (1989). Briefly, frozen cortex was homogenized in 10 mM Tris-HCl buffer containing 0.32 M sucrose and 0.5 mM EDTA at pH 7.40. The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was centrifuged again at 48,000 × *g* for 60 min. The resultant pellet was resuspended in 10 mM HEPES–1 mM EDTA buffer, pH 7.0, incubated at 32°C for 30 min, and centrifuged at 40,000 × *g* for 45 min; this step was repeated twice. Membranes were washed with HEPES buffer and centrifuged two additional times without incubation. The protein concentration was determined using the method of Lowry et al. (1951) and adjusted to a final concentration of 1 mg/ml with HEPES–EDTA buffer.

^{3}H-MK-801 saturation binding assays were performed in a 200 μl total volume containing 75 μg of membrane protein, 100 μM glutamate, and 100 μM glycine in HEPES–EDTA buffer as previously described (Hoffman et al., 1994). Nonspecific binding was determined in the presence of 10 μM unlabeled MK-801. ^{3}H-MK-801 was added at concentrations ranging from 0.5 to 50 nM. The reaction mixture was incubated at 32°C for 3 hr and terminated by the addition of excess ice-cold HEPES buffer. Samples were filtered through glass fiber filters that were washed with additional cold buffer. Total radioactivity in the filters was counted in an LKB-Wallac (Gaithersburg, MD) Rackbeta 1209 scintillation counter with an efficiency of 65% for ^{3}H.

Data analysis. Scatchard plots were constructed using the results of the saturation binding assays. B_{max} (apparent number of receptors) and K_d (dissociation constant) were determined from the Scatchard plots. Results among the experimental groups were compared using a one-way ANOVA; if the ANOVA demonstrated significance, pairwise comparisons were made using the unpaired Student's *t* test with Bonferroni's correction. A *p* value of < 0.05 was considered significant.

RESULTS

A total of 30 pregnant ewes were studied, 10 at 88 d (four placebo, six treated), 9 at 120 d (four placebo, five steroid-treated), and 11 at 136 d (six placebo, five steroid-treated). However, complete results of ^{3}H-MK-801 binding could not be obtained in the samples from the corticosteroid-treated fetuses at 88 d gestation because of technical difficulties resulting from insufficient available tissue. Twelve lambs were studied after treatment with corticosteroids (six low-dose, six high-dose), with five serving as placebo-treated controls. ^{3}H-MK-801 binding assays were also performed using cerebral cortex from eight of the ewes (five placebo, three steroid-treated). There was no significant effect of corticosteroid treatment on arterial blood gases or cardiovascular function in fetuses. However, in lambs, low-dose dexamethasone was associated with a higher $PaCO_2$, lower PaO_2, and lower heart rate compared to lambs that received either placebo or high-dose dexamethasone (Table 1). Serum cortisol and blood glucose concentrations in fetuses did not change significantly with gestational age, nor were they affected by *in utero* exposure to dexamethasone (Fig. 1). Blood glucose concentrations in lambs were significantly higher than fetal levels, and blood glucose concentrations in lambs treated with low-dose dexamethasone were lower than levels in either placebo-treated or high-dose dexamethasone-treated animals (Table 1). Cortisol levels were significantly higher in the placebo-treated lambs compared to the fetuses. Postnatal treatment with dexamethasone resulted in a significant dose-related decrease in serum cortisol levels. Cortisol levels in the ewes were not altered by maternal corticosteroid administration.

Representative Scatchard plots of data obtained from the ^{3}H-MK-801 binding assays in 120 and 136 d fetuses, 5-d-old lambs, and adult ewes are shown in Figure 2. B_{max} (apparent number of receptors) increased during development, reaching a maximum value in 5-d-old lambs (Table 2). The value in adult ewes was significantly lower than the values in 120 and 136 d fetuses and in 5-d-old lambs. However, the affinity of the receptor ion channel for ^{3}H-MK-801, as indicated by the K_d, did not change significantly with maturation.

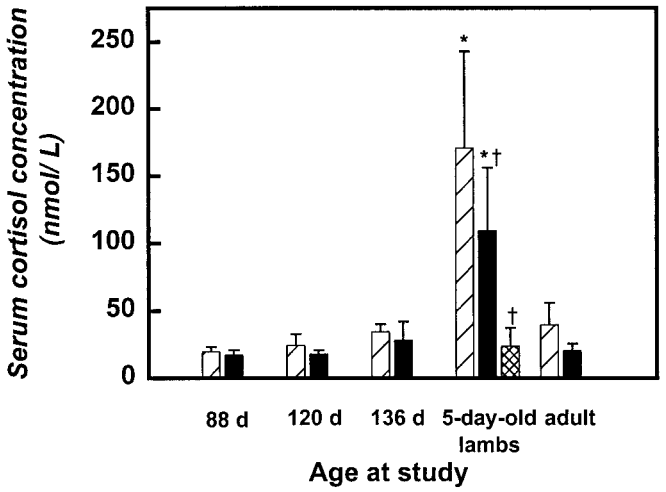

Figure 1. Serum cortisol levels in fetal, newborn, and adult sheep after treatment with placebo or dexamethasone. Dexamethasone treatment in fetuses and ewes consisted of 6 mg/kg intramuscularly given to pregnant ewes every 12 hr for four doses. Newborn lambs received either 0.01 mg/kg (low dose) or 0.25 mg/kg (high dose) intramuscularly every 12 hr for four doses. Placebo-treated, *Bars* with *diagonal lines*; dexamethasone-treated (all fetuses and low-dose lambs), *filled bars*; high-dose lambs, *hatched bars*; $*p < 0.05$ versus placebo- and dexamethasone-treated fetuses; $\dagger p < 0.05$ versus placebo-treated lambs.

Dexamethasone treatment for 48 hr resulted in a significantly lower B_{max} in 5-d-old lambs compared to age-matched saline-treated animals regardless of the dose of corticosteroids administered. K_d decreased by 20% in both groups of steroid-treated lambs, suggesting a corticosteroid-induced increase in receptor affinity (Table 1). In contrast, there were no differences in ^{3}H-MK-801 binding characteristics in fetal cerebral cortical membranes after maternal corticosteroid administration at any gestational age compared to age-matched controls. Pregnant ewes treated with dexamethasone also did not exhibit any differences in ^{3}H-MK-801 binding compared to saline-treated controls.

DISCUSSION

In the present study, we found that the apparent number of NMDA receptor MK-801 binding sites (B_{max}) increased during development in the sheep. B_{max} in both the near-term fetus (136 d gestation) and the newborn lamb was significantly higher than in the adult, with the maximum value observed at 5 d postnatal age (Fig. 3). In contrast, the affinity of the receptor ion-channel binding site, as indicated by the K_d for ^{3}H-MK-801, did not change significantly during gestation. These results are consistent with results reported in the guinea pig for both ^{3}H-glutamate binding and ^{3}H-MK-801 binding (Mishra and Delivoria-Papadopoulos, 1992; Abdollah and Brien, 1995). Studies using autoradiography have demonstrated that NMDA-dependent ^{3}H-glutamate binding in the fetal sheep peaks at 135 d gestation and remains at the peak level in the first few postnatal days (Anderson et al., 1999). Combined with our data, these results suggest that there is a delay between the appearance of the receptors in ovine brain and the development of full function of the receptor-ion channel complex as demonstrated by the ability of MK-801 to bind to the open ion channel. In the human fetus, the number of NMDA receptors as determined by NMDA-dependent ^{3}H-glutamate binding was reported to increase at 22 weeks of gestation compared to the value at 16 weeks; by 24–26 weeks, the number of receptors decreased, but remained higher than adult values (Lee and Choi, 1992). In infants, ^{3}H-MK-801 binding increased between term and 26 weeks postnatal age. Activation of the receptor by glutamate and glycine also increased with increasing postnatal age, indicating continued modification of the receptor during brain maturation (Slater et al., 1993). The apparent increase in NMDA receptor number during brain development is most likely linked to the morphological and functional changes in cerebral cortex, including synaptogenesis, dendritic arborization, and remodeling of synaptic connections that occur during the late fetal and early neonatal periods (Cotman et al., 1994; Brooks et al., 1997). Cell migration and survival as well as synapse maturation and long-term potentiation during brain development appear to be regulated by changes in intracellular Ca^{2+}, in large part mediated by NMDA receptor activity (Pontzer et al., 1990; Robinson and Reed, 1992; Vaccarino et al., 1992; Asztély and Gustafsson, 1996).

We found that corticosteroid administration resulted in age-specific effects on the developmental changes in the cerebral cortical NMDA receptor. In newborn lambs, 48 hr of exposure to dexamethasone significantly decreased the apparent number of NMDA receptors and was associated with a 20% increase in the affinity of the MK-801 binding site. This was true even when extremely low doses of dexamethasone were used (0.01 mg/kg every 12 hr, total dose 0.04 mg/kg). However, *in utero* exposure to dexamethasone had no effect on the B_{max} or K_d for MK-801 at 120 or 136 d of gestation in the sheep fetus. Although it is not possible to determine from our data whether the decrease in B_{max} represents acceleration or retardation of the normal developmental changes in NMDA receptor number, it is likely that steroid exposure alters the characteristics of the NMDA receptor by enhancing receptor maturation, triggering the developmental decrease in B_{max} toward the adult value at an earlier postnatal age.

The ontogeny of cerebral cortical corticosteroid receptors is not well described. In the preterm fetal lamb, corticosteroid receptors may not be developed enough to allow a response to the dose of corticosteroids used for antenatal treatment. Myocardial β-adrenergic receptor function in fetal lambs at 120–130 d gestation was not altered by exposure to either thyroid hormones or betamethasone; in contrast, the density of the β-adrenergic receptors increased in treated newborn lambs (Padbury et al., 1986; Berry et al., 1997). However, comparable doses of dexamethasone did alter blood–brain barrier function in fetal sheep at 120 d gestation (Stonestreet et al., 1999), and antenatal treatment with steroids increased adenylate cyclase activity in immature myocardium (Stein et al., 1993). Thus, the differences observed could reflect a cell-specific difference in the ontogeny of steroid receptor function. Another possibility is that there are specific repressor mechanisms that prevent activation of steroid receptors (Tseng et al., 1995) or inhibit signal transduction in some cell populations after activation of steroid receptors.

Corticosteroids could alter the apparent number of NMDA receptors via several mechanisms. The actual number of receptors could decrease because of downregulation of transcription or translation of the genes coding for the receptor subunits. Previous studies on the effect of corticosteroids on gene expression have shown that corticosteroids may mediate gene transcription by binding to specific cytoplasmic and nuclear membrane receptors (Tanaka et al., 1995). However, the specific effects of corticosteroid exposure on the ionotropic glutamate receptors have not been well characterized. Exposure of adult rats to corticosterone at doses associated with a corticosteroid-like effect increased the B_{max} of the NMDA receptor for MK-801, whereas the K_d for MK-801 binding was unchanged (Weiland et al., 1997). Similarly, exposure to dexamethasone increased the B_{max} for NMDA-dependent ^{3}H-glutamate binding (Nair et al., 1998). Other studies have found no changes in either NMDA receptor subunit mRNA levels or ^{3}H-glutamate binding after corticosterone exposure (Supko and Johnston, 1994; Kew et al., 1998). A decrease in NMDA receptor gene expression or apparent receptor number following treatment with corticosteroids has not been reported in adult animals. However, steroid exposure during development may produce different effects than those observed in mature animals.

Exposure to dexamethasone could also modify existing receptors or the synaptic membrane surrounding the receptors, altering the conformation of the receptor such that the accessibility of the binding site for ^{3}H-MK-801 is decreased. Corticosteroids may bind directly to membranes and alter cell function via nongenomic

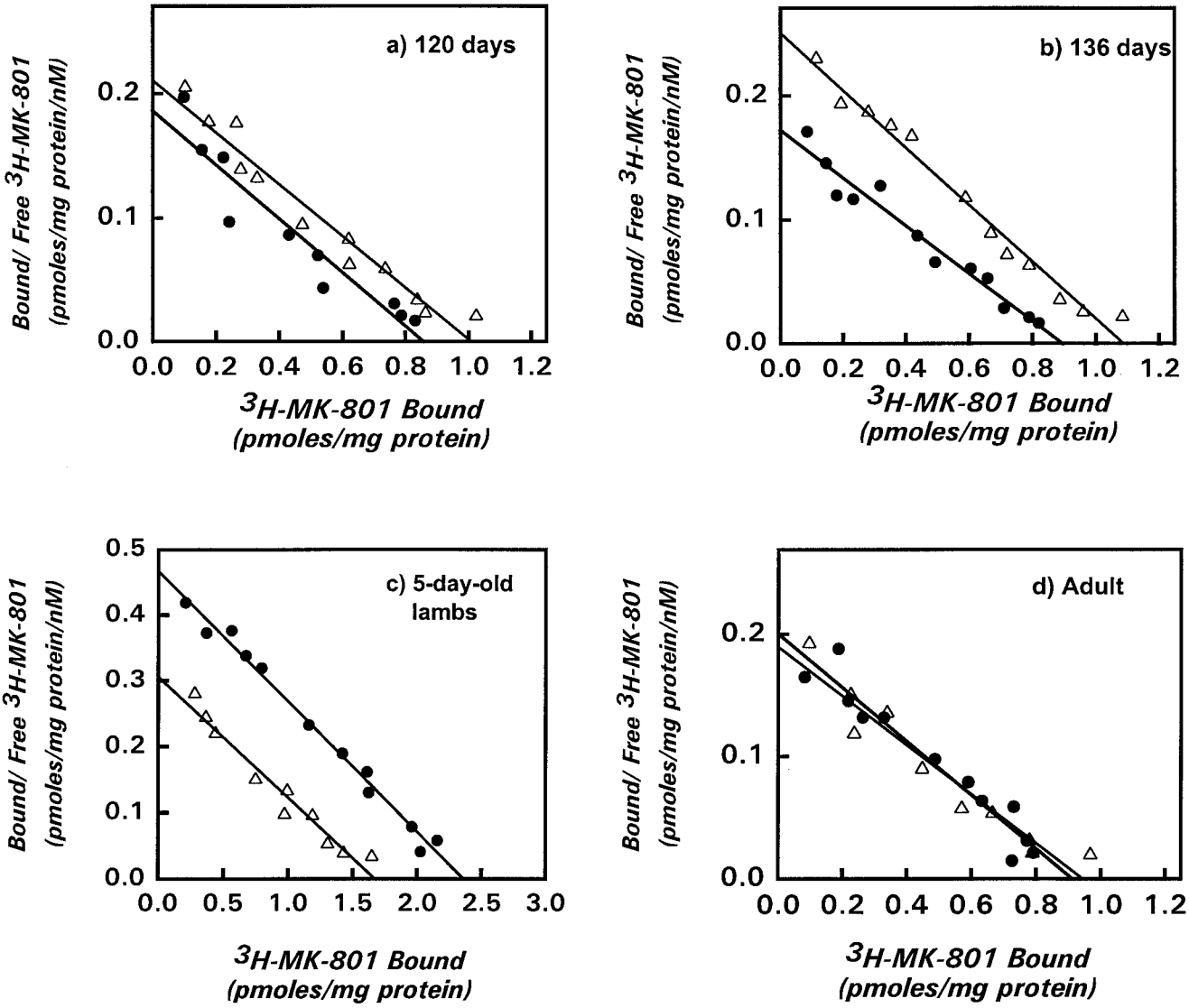

Figure 2. Representative Scatchard plots of results of ^{3}H-MK801 binding in cerebral cortical membranes prepared from fetal lambs at 120 and 136 d gestation, 5-d-old newborn lambs, and adult ewes. Placebo-treated, *Filled circles*; dexamethasone-treated, *open triangles* (for details of treatment regimen, see legend for Fig. 1.).

Table 2. ^{3}H-MK-801 binding in sheep brain

Study group (n)	B_{max} (pmol/mg protein)		K_d (nM)	
	Placebo	Dexamethasone	Placebo	Dexamethasone
88 d gestation	0.67 ± 0.09 (4)*,**	—	5.07 ± 0.42	—
120 d gestation	0.97 ± 0.12 (4)**	0.96 ± 0.14 (5)	5.24 ± 0.55	4.85 ± 0.42
136 d gestation	1.44 ± 0.20 (6)**	1.48 ± 0.19 (5)	5.20 ± 1.09	4.50 ± 0.97
5-d-old lambs	2.17 ± 0.18 (5)	LD: 1.65 ± 0.08 (6)**	5.40 ± 0.40	4.41 ± 0.40**
		HD: 1.62 ± 0.07 (6)**		4.03 ± 0.32**
Ewes	1.07 ± 0.09 (5)	0.98 ± 0.04 (3)	4.49 ± 0.38	4.62 ± 0.65

*Mean ± SD (*n*).

**$p < 0.05$ vs placebo-treated lambs.

effects such as alterations in transmembrane calcium fluxes, membrane fluidity, and protein phosphorylation (Sze and Yu, 1995; Golden et al., 1998; Whiting et al., 1998). Such changes in membrane structure and function could lead to the observed changes in the B_{max} and K_d for MK-801. Corticosteroids could also decrease apparent NMDA receptor number by increasing receptor degradation, although this is less likely because this effect has not been previously reported in other models following steroid administration. Activation of corticosteroid receptors may also increase glutamate dehydrogenase activity, leading to a decrease in extracellular glutamate levels (Hardin-Pouzet et al., 1996). However, decreased extracellular glutamate would be expected to increase, rather than decrease, the number of NMDA receptors, as was the case in the present study.

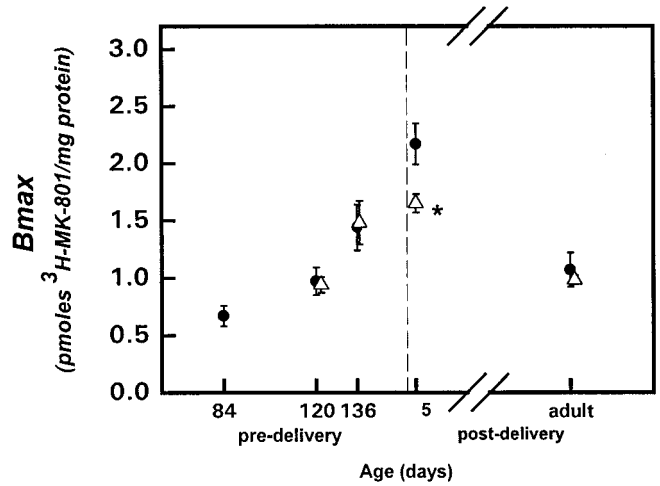

Figure 3. Developmental pattern of NMDA receptor B_{max} (apparent receptor number) and effect of dexamethasone treatment in the sheep as determined by ^{3}H-MK801 binding. Placebo-treated, *Filled circles*; dexamethasone-treated, *open triangles*; $^*p < 0.05$ versus placebo-treated.

Antenatal administration of corticosteroids accelerates maturation of multiple organ systems and appears to decrease the incidence of intraventricular hemorrhage in preterm infants, suggesting that corticosteroids exert a neuroprotective effect on immature brain. The developmental increase in the number of NMDA receptors may put the near-term sheep fetus and newborn lamb at increased risk for excitotoxic brain injury compared to the early gestation fetus or adult. A decrease in the number of NMDA receptors could be neuroprotective under conditions associated with excitotoxicity, such as hypoxia–ischemia, because cellular Ca^{2+} influx via the NMDA receptor would likely be reduced. However, we observed a corticosteroid-mediated change in the NMDA receptor only in the term newborn lamb; no effect of dexamethasone treatment was observed in fetal lambs at levels of brain development similar to the level of development found in preterm infants at risk for intraventricular hemorrhage. Thus, it is likely that the decrease in the incidence of intraventricular hemorrhage seen in preterm infants exposed to corticosteroids *in utero* results from a mechanism other than modification of the NMDA receptor, such as alteration of blood–brain barrier permeability or neuronal ion transport.

NMDA receptor activity appears to be required for synaptogenesis and long-term potentiation, two important processes during normal brain development. Thus, early downregulation of NMDA receptor number could alter patterns of synapse formation leading to later behavioral or cognitive abnormalities. Although we did not investigate specific functional or structural consequences of the changes we observed in the NMDA receptor MK-801 site binding characteristics after treatment with dexamethasone, studies in other animal models suggest that alterations in MK-801 binding characteristics occur in association with other significant alterations in brain structure or function. For example, exposure of immature brain to ethanol is associated with a decreased B_{max} for ^{3}H-MK-801 (Valles et al., 1995) as well as altered NMDA receptor-mediated Ca^{2+} influx (Lee et al., 1994) and decreased synaptic density (Tanaka et al., 1991). Blockade of the NMDA receptor ion channel, in effect a functional decrease in the B_{max} for MK-801, is also associated with decreased synaptic density (Butler et al., 1999). Although direct causal relationships cannot be assumed from these studies, the results suggest that the presence of a decrease in B_{max} is associated with altered brain structure and function. As previously discussed, a relatively low dose of dexamethasone was sufficient to decrease serum cortisol levels and induce changes in the cerebral cortical NMDA receptor in the newborn lamb, suggesting that even limited exposure to corticosteroids during a critical period could have significant effects on the developing brain. Further studies are needed to evaluate the long-term effects of corticosteroid treatment during development on brain structure and function.

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