0031-3998/03/5306-0956
PEDIATRIC RESEARCH
Vol. 53, No. 6, 2003
Copyright © 2003 International Pediatric Research Foundation, Inc.
Printed in U.S.A.

Changes in 5α-Pregnane Steroids and Neurosteroidogenic Enzyme Expression in the Perinatal Sheep

PHUONG N. NGUYEN, SARAID S. BILLIARDS, DAVID W. WALKER, AND JONATHAN J. HIRST

Fetal and Neonatal Research Group, Department of Physiology, Monash University, Clayton, Victoria 3800 Australia

ABSTRACT

Pregnane steroids have sedative and neuroprotective effects on the brain as a result of interactions with the steroid-binding site of the $GABA_A$ receptor. To determine whether the fetal brain is able to synthesize pregnane steroids *de novo* from cholesterol, we measured the expression of cytochrome P450 side-chain cleavage (P450scc) and 5α-reductase type II (5αRII) enzymes in fetal sheep from 72 to 144 d gestation (term ~147 d) and in newborn lambs at 3 and 19-26 d of age. Both P450scc and 5αRII expression was detectable by 90 d gestation in the major regions of the brain and also in the adrenal glands. Expression increased with advancing gestation and was either maintained at fetal levels or increased further after birth. In contrast, the relatively high content (200-400 pmol/g) of allopregnanolone (5α-pregnan-3α-ol-20-one), a major sedative 5α-pregnane steroid, present throughout the brain from 90 d gestation to term, was reduced significantly (<50 pmol/g) immediately after birth. These results suggest that although the perinatal brain has the enzymes potentially to synthesize pregnane steroids *de novo* from cholesterol, either the placenta is a major source of these steroids to the brain or other factors associated with intrauterine life may be responsible for high levels of allopregnanolone production in the fetal brain until birth. **(*Pediatr Res* 53: 956–964, 2003)**

Abbreviations

AP, allopregnanolone, 5α-pregnan-3α-ol-20-one
P450scc, P450 side chain cleavage enzyme
5αRII, 5α-reductase type II enzyme
PMC, primary motor cortex
$GABA_A$, γ-aminobutyric acid/benzodiazepine receptor
5α-DHP, 5α-dihydroprogesterone

Neuroactive steroids such as allopregnanolone (AP) are potent neuromodulators that modify the excitability of the CNS by interaction with the γ-aminobutyric acid/benzodiazepine receptor-chloride ionophore ($GABA_A$ receptor). In the adult AP is a positive allosteric modulator of the $GABA_A$ receptor with potent anxiolytic (1, 2), anticonvulsant (3, 4), sedative/hypnotic, and anesthetic effects (5) on behavior. Prenatally, neuroactive steroids have been shown to suppress fetal activity in late gestation and seem to have a role in maintaining the low level of arousal-like behavior that typifies fetal life (6, 7). This interaction between AP and the $GABA_A$ receptor complex is responsible for the major pharmacologic actions of AP and is distinct from the genomic effects exerted by other steroids such as progesterone (8). In the brain, neuroactive steroids such as AP are synthesized *de novo* from cholesterol, but a proportion of the pool of steroids may be derived from precursors in blood that enter the brain across the blood-brain barrier (9–11). Thus, peripheral steroidogenesis may influence neuroactive steroid content in the brain.

The cytochrome P450 side-chain cleavage enzyme (P450scc) catalyzes the irreversible conversion of cholesterol to pregnenolone on the inner side of the mitochondrial membrane (12). The primary site of pregnenolone synthesis in the brain seems to be in oligodendrocytes and astrocytes, with significantly less synthesis occurring in neurons (13–16). 5α-reductase catalyzes the irreversible conversion of progesterone to 5α-dihydroprogesterone (5α-DHP), the immediate precursor for AP formation. Two isoforms of the 5α-reductase enzyme have been cloned—both catalyze the same reaction but have different biochemical properties and ontogeny-related expression in the brain (17, 18). In rat and sheep, the type II isoform is expressed toward the end of gestation through to the early neonatal period (5, 19, 20) and is present in neurons, astrocytes, and glia at these times. The type I isoform is mainly expressed in the adult brain (21); however, localization remains unknown.

Our recent studies indicate that the fetal and newborn sheep CNS is sensitive to neurosteroids. Administration of proges-

Received July 2, 2002; accepted January 13, 2003.
Correspondence: Dr. Jonathan Hirst, Department of Physiology, Monash University, Clayton Victoria 3800, Australia; e-mail: j.hirst@med.monash.edu.au
Supported by a Monash University Faculty of Medicine Postgraduate Scholarship to P.N.N., a National Health and Medical Research Council Dora Lush Postgraduate Scholarship to S.S.B., and National Health and Medical Research Council of Australia Grant No. 143503 to J.J.H. and D.W.W.

DOI: 10.1203/01.PDR.0000064905.64688.10

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited

terone to the pregnant ewe or of pregnanolone to the fetus produced behavioral effects consistent with $GABA_A$-mediated actions (22). Inhibition of 5α-reductase activity with finasteride produced behavioral effects consistent with reduced activation of $GABA_A$ receptors (7). These observations suggest that 5α-reduced steroids produced in late gestation may regulate the activity of the fetal brain. The aims of this study were to examine the developmental changes in the expression of the two key steroidogenic enzymes, P450scc and 5αRII, in the fetal and newborn sheep brain and to determine AP content in the brain before and after birth so as to elucidate whether the concentrations of these steroids are sufficient to modulate $GABA_A$ receptors in the fetus.

METHODS

All procedures were conducted in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes of the National Health and Medical Research Council and had received prior approval from the Monash University Standing Committee on Ethics in Animal Experimentation.

Animals. Pregnant Border Leicester-Merino crossbred ewes carrying fetuses of known gestational age were used. The ewes were housed under 12-h light:dark cycle conditions in individual cages and were fed once daily between 0900 h and 1200 h with water available *ad libitum*. The ewes and fetuses were killed by injection of an overdose of sodium pentobarbitone (130 mg/kg i.v.) given to the ewe at 72 (n = 4), 90 (n = 4), 113 (n = 4), 126 (n = 4), 132–134 (n = 4), 137–138 (n = 4), 140 (n = 4), and 144 d (n = 4) of gestation (term ~147 d). Lambs were obtained from ewes maintained under the same conditions and were killed at 3 (n = 4) and 19–26 d (n = 4) of postnatal age. Each brain was immediately removed, and blocks corresponding to the following regions were collected: cerebellum; hippocampus; medulla; midbrain; pons; hypothalamus/thalamus; and primary motor (PMC), frontal, parietal, occipital, and temporal cortex. Fetal adrenal glands were also collected. All tissue was frozen in liquid nitrogen and stored at −70°C.

P450scc and 5αRII Antisera. Polyclonal antipeptide antibodies were prepared in New Zealand White rabbits following standard protocols (23). Antibodies against ovine P450scc were raised in three rabbits against a synthetic peptide corresponding to the amino acids 501–515 of the enzyme deduced from the cDNA sequence (24). Antibodies against 5αRII were raised in another three rabbits against a synthetic peptide corresponding to the amino acids 227–246 of the enzyme as deduced from the cDNA sequence (25). Both peptides were synthesized using Fmoc solid-phase peptide synthesis and conjugated to the carrier protein Keyhole Limpet Hemocyanin using maleimidocaproyl-N-hydroxysuccinimide (26). The purity of the synthesized peptides was verified by reverse-phase HPLC and identified by ion spray mass spectrometry and were of 80% and 94% purity, respectively. Either peptide was mixed with Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO, U.S.A.) and injected s.c. into six different flank regions of New Zealand White rabbits following standard protocols (23). A booster injection with peptide suspended in Freund's incomplete adjuvant (Sigma Chemical Co.) was administered 3 wk after the initial immunization. A further booster was given 3 wk after the first booster dose. Serum was collected from an ear artery bleed before each immunization and 10–14 d after each booster injection. Antibody content from each bleed was assessed using an ELISA assay as previously described (27).

Immunoblotting. Cytochrome P450scc and 5αRII expression in the ovine brain and adrenal glands was determined by Western immunoblotting as previously described (28). In brief, frozen samples (~0.1 g) from each brain region or adrenal glands were powdered on solid CO_2 using a mortar and pestle and then homogenized in 1.5 mL of ice-cold homogenizing buffer [50 mM Tris-HCl, 2 mM EDTA, and 0.05% (vol/vol) Nonidet P-40 and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4)]. The homogenate was centrifuged, and the supernatant was then concentrated by precipitation with 40% saturation of ammonium sulfate. The pellet was resuspended in distilled water, and protein content was determined in an aliquot according to the method of Bradford (29) using BSA as a standard. Protein (15 μg) was separated using 10% SDS-PAGE and then transferred to 0.2 μM polyvinylidene difluoride membranes (Osmonics, Westborough, MA, U.S.A.) by electroblotting. Membranes were then blocked in TBST [25 mM Tris, 14 mM sodium chloride, 0.2% (vol/vol) Tween-20 (pH 7.4)] containing 5% (wt/vol) skim milk powder. The membrane was incubated with a 1:3000 dilution of either the P450scc or 5αRII antibody in TBST and then washed with TBST and incubated with a 1:3000 dilution of a horseradish peroxidase–conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark). The immune complexes were visualized by chemiluminescence using the Amersham ECL detection system (Amersham, Buckinghamshire, England). Chemiluminescence on membranes was captured using BioMax ML autoradiograph film (Eastman Kodak, Rochester, NY, U.S.A.). Immunoblots were scanned and analyzed using ImageQuaNT software (Amersham, Buckinghamshire, England). The densities of the bands were determined and were individually corrected for background by subtracting the density of the blank background area immediately below each band.

The specificity of each antiserum was assessed by using primary antiserum that had been preabsorbed with 10 μg of the appropriate purified antigen for 24 h at 37°C before use (Fig. 1). Preimmune serum was also incubated to assess the degree of nonspecific binding (Fig. 1)

To allow the comparison of expression at all gestational and neonatal ages for a particular brain region, we loaded samples for each region at each fetal or neonatal age onto two 20-lane gels that were run under identical conditions. A brain sample from a late-gestation fetus was used as a reference/positive control and was loaded on the first lane of each of the two gels. The remaining lanes of the two gels were loaded with samples from the four animals in each fetal (72, 90, 113, 126, 137–138, and 144 d gestation) and neonatal (3 and 19–26 d) age group.

Neurosteroid RIA. Neurosteroids were extracted from brain or adrenal tissue and plasma by a modification of the method of Barbaccia *et al.* (30) as previously described (31). In brief, frozen tissue (~0.1g) from each brain region or adrenal gland

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited

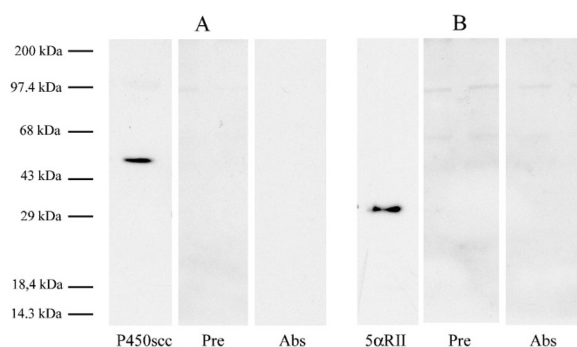

Figure 1. Validation of ovine P450scc (*A*) and 5αRII (*B*) antisera raised in rabbits. There was a strong band at the 50 kD position for P450scc when the blots were incubated with the ovine P450scc antibody (*A*) and a strong band at the 26 kD position for 5αRII when the blots were incubated with the 5αRII antibody (*B*). No bands were observed when the blots were incubated with preimmune sera (Pre) or when the blots were incubated with antisera that had been preabsorbed with their respective antigen (Abs).

was powdered on dry ice and then extracted with 50% methanol containing 1% acetic acid. After centrifugation, the supernatant was collected and the pellet was reextracted twice with the above solvent. The supernatants were combined and applied to Sep-Pak C-18 cartridges (Waters Corp, Milford, MA, U.S.A.) previously equilibrated with absolute methanol, followed by 50% methanol and then 50% methanol + 1% acetic acid. Plasma samples (0.1 mL) were mixed with 50% methanol + 1% acetic acid (1:10) before loading on the Sep-Pak cartridges. After the samples were loaded, the cartridges were washed with 50% methanol + 1% acetic acid, followed by 50% methanol. The steroids were then eluted with 100% methanol, the collected fractions were dried under N_2, and the steroids were resuspended in 1.0 mL of assay buffer (0.1 M PBS, pH 7.0). Two additional samples of brain, adrenal glands, and plasma containing tritium-labeled steroid were extracted in parallel with each extraction run to estimate extraction efficiency. Recovery of AP, progesterone, and pregnenolone from plasma was 75%, 91.75%, and 91.75%, respectively ($n = 1$ extraction). Recovery from brain was 77% ± 6%, 66% ± 4%, and 75% ± 6%, respectively ($n = 3$ extractions). Recovery from adrenal glands was 95% ($n = 1$), 88% ($n = 1$), and 90% ($n = 3$), respectively. Corrections for losses during extraction were included in final calculations.

AP was measured by specific RIA as previously described (31). Briefly, AP standard was purchased from Sigma Chemical Co. Aldrich and was used to prepare a standard curve ranging from 0 to 3.1 pmol/tube. Pregnan-3α-ol-20-one, 5α=[9,11,12,3H(N)] (45 Ci/nmol, ^{3}H-AP) was obtained from Geneworks (Adelaide, Australia). A polyclonal antisera raised in sheep against AP carboxymethyl ether coupled with BSA was purchased from Dr. R.H. Purdy (Department of Psychiatry, Veterans Administration Hospital, San Diego, CA, U.S.A.), which has been previously characterized (32). The cross-reactivities for the AP antisera were 6.5% for 3α-hydroxy-5β-pregnan-20-one (progesterone); 0.7% for pregn-4-ene-3,20-dione; and <0.1% for 5α-pregnane-3,20-dione, 5β-pregnane-3,20-dione, 20β-hydroxy-5α-pregnan-3-one, 5α-pregnane-3α,20α-diol, 3β-hydroxy-5α-pregnan-20-one, 5β-pregnane-3α,20α-diol, 3βhydroxy-5β-pregnan-20-one, and 5α-pregnane-3β,20αa-diol (32). Extracts from brain, adrenal glands, and plasma that contained AP in the range of concentrations found in respective samples serially diluted in parallel with the standard curve. The limit of detection for AP was 0.19 ± 0.03 pmol/tube ($n = 3$), and the interassay coefficients of variance were as 5% and 9%, respectively.

Pregnenolone and progesterone were measured by specific RIA as previously described (31). The pregnenolone antibody was purchased from ICN Biomedicals (Seven Hills, NSW, Australia). The cross-reactivities for pregnenolone were 3.1% for progesterone; <1.0% for AP; and <0.1% for 5α-dihydroprogesterone, deoxycorticosterone, 17α-hydroxypregnenolone, 20α-dihydroprogesterone, cortisol, dihydroepiandrosterone, 11-deoxycortisol, corticosterone, 5α-dihydrotestosterone, estradiol-17β, and 20β-dihydroprogesterone. Brain extracts containing pregnenolone at similar concentrations to that found in the brain were found to be serially diluted in parallel with the standard curve. The limit of detection for pregnenolone was 0.09 ± 0.02 pmol/tube ($n = 3$), and the interassay coefficients of variance were 5% and 16%, respectively. The progesterone antibody was provided by Dr. J. Malecki (Bairnsdale, Victoria, Australia), which also has been previously characterized (33). The cross-reactivities for the progesterone antisera were 43.8% for 11α-hydroxy-progesterone; 15.9% for 5α-pregnane,3α-ol,20-one; 10.0% for 5β-pregnane,3α-ol,20-one; and <1.0% for corticosterone, 17α-hydroxy-progesterone, 20α-hydroxy-pregnane,3-one, 11-deoxycortisol, 5α-pregnane,3β-ol-2-one, 5α-pregnane,3α,17α-diol-20-one, 5β-pregnane,3α,17α,20α-triol-20-one, 5β-pregnane,3α,17α,20α-triol, dehydroepiandrosterone, and cortisol. The limit of detection for progesterone was 0.12 ± 0.02 pmol/tube ($n = 3$), and the intra- and interassay coefficients of variance were 8% and 19%, respectively.

Statistical Analysis. Data are shown as mean ± SEM. All data were analyzed using statistical software (SPSS Version 9.0; SPSS, Inc., Chicago, IL, U.S.A.). One-way ANOVA followed by Fischer's LSD test was used to determine significance between each age group. $P < 0.05$ was considered to be statistically significant.

RESULTS

Specificity of Antibodies

All three antisera raised against the P450scc peptide showed no cross-reactivity with 5αRII as tested by ELISA and Western immunoblotting, and produced a single band on immunoblots of fetal brain tissue at 50 kD (Fig. 1). The antiserum with the highest titer and specificity was selected for subsequent studies. Two of the antisera raised against the 5αRII peptide produced a single band at 26 kD (the expected size of ovine and human 5αRII), and the antiserum with the highest titer was selected for subsequent studies (Fig. 1). A third antiserum produced two bands at 23 kD and 26 kD, representing both 5αRI and 5αRII, respectively, and was not used. Preimmune sera did not react to either of the two synthesized peptides. Neither of the two selected antisera showed detection of any bands on immunoblots when preabsorbed by incubation with their respective antigen (Fig. 1). These two antibodies were

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited

used for the subsequent studies of P450scc and 5αRII expression in fetal and newborn tissue.

Expression of P450scc and 5αRII

Regional P450scc and 5αRII Expression in the Brain. The relative abundance of P450scc and 5αRII expression in the different brain regions was assessed at two fetal (90 and 126 d gestation) and two postnatal ages (3 and 26 d old) is shown in Figure 2*A* and 2*C*. Expression is shown in a single representative animal at two gestational (90 and 126 d gestational age) and two postnatal ages (3 and 26 d of postnatal age). As shown in Figure 2, all regions at each gestational or postnatal age were examined on individual immunoblots; however, a sample from a late-gestational fetus was included in each blot as a positive control and was loaded as a reference to compare between gels. At 90 d gestation, the highest relative expression of both P450scc and 5αRII occurred in the medulla, midbrain, and pons (Fig. 2, lanes 3, 4, and 5). The high expression was also present at 126 d gestation and 3 d postnatal. Expression in the hypothalamus/thalamus (lane 6) and all regions of the cortex examined (lanes 7–11) was low relative to the brain stem regions at 90–126 d gestation and seemed to increase postnatally. Figure 2*C* and 2*D* shows P450scc and 5αRII expression, respectively, in all brain regions at 126 d gestation and indicates that the differences observed between brain regions were consistently seen in four different fetuses.

Gestational Changes in P450scc and 5αRII Expression in the Brain. For determining the changes in expression of P450scc and 5αRII with developmental age, gels that contained samples of a particular brain region for each fetal or neonatal age were run. In general, expression of both enzymes increased with advancing gestational age, with the highest fetal levels occurring in the 126-d or 137- to 138-d gestational age groups (Fig. 3). For most areas of the brain, enzyme expression was increased further in the postnatal groups, the exceptions being P450scc expression in the medulla, which decreased slightly after birth, and 5αRII expression in the hypothalamus/thalamus and PMC, where postnatal levels were similar to those found in the fetal brain. The cerebellum was most notable

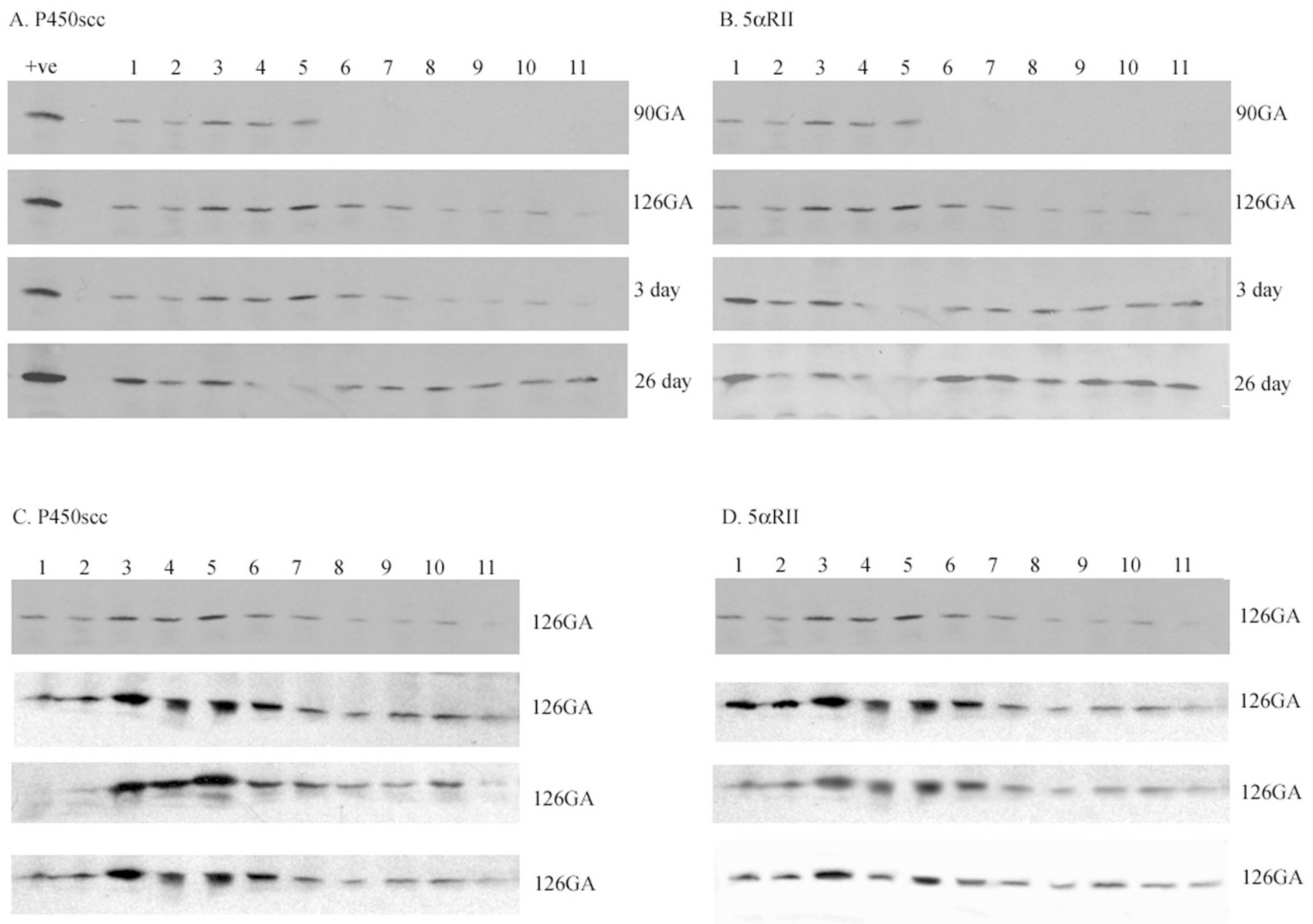

Figure 2. Expression of P450scc (*A* and *C*) and 5αRII (*B* and *D*) in the fetal and neonatal brain. *A* and *B*, Expression in fetal (90 and 126 d gestation) and neonatal (3 and 19–26 d of postnatal age) brain from a representative animal at each age. *C* and *D*, Expression in four fetuses at 126 d gestational age. Lanes marked indicate the following regions: 1, cerebellum; 2, hippocampus; 3, medulla; 4, midbrain; 5, pons; 6, hypothalamus/thalamus; 7, PMC; 8, frontal cortex; 9, occipital cortex; 10, parietal cortex; and 11, temporal cortex. Expression of both P450scc and 5αRII was found to be highest in the brain stem (medulla and pons) throughout gestation and in the neonate.

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited

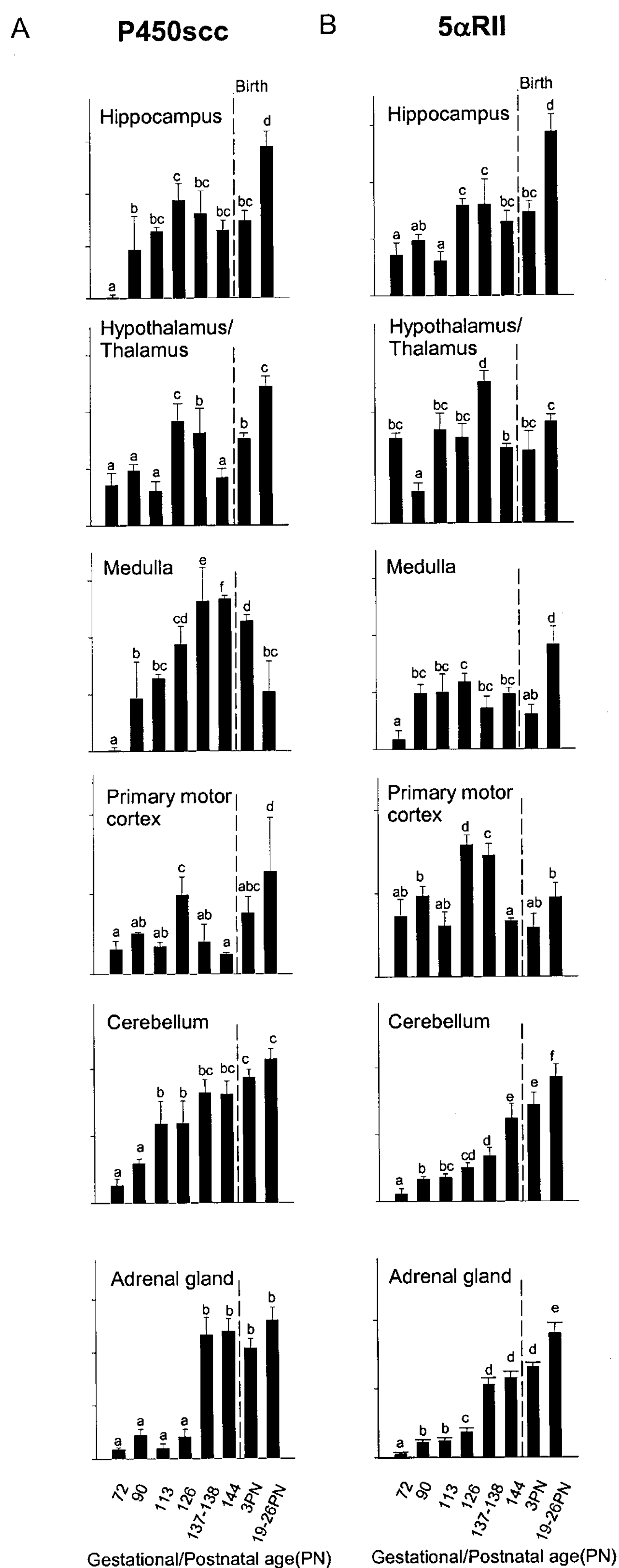

Figure 3. Densitometry values in arbitrary units of P450scc (*A*) and 5αRII (*B*) expression in the hippocampus, hypothalamus/thalamus, medulla, PMC, cerebellum, and adrenal glands of fetuses between 72 and 144 d gestation and lambs at 3 and 19–26 d of postnatal age. Expression in most areas was increased during gestation and rose further after birth. Each bar represents the mean ± SEM for four animals. Different lower case lettering indicates significance difference, $p < 0.05$.

for the progressive increase of expression of both enzymes from midgestation to at least 3 wk after birth (Fig. 3).

Gestational Changes in P450scc and 5αRII Expression in the Adrenal Glands. Both P450scc and 5αRII expression was present in the adrenal glands from 72 d gestation. P450scc expression increased significantly between 126 and 137–138 d gestation, and the postnatal levels were similar to those found in the late-gestation fetus (Fig. 3). 5αRII expression also increased significantly between 126 and 137–138 d gestation, and there was a further significant increase between 3 d and 19–26 d after birth (Fig. 3).

Brain Steroid Content

AP was detected in the hippocampus, hypothalamus/thalamus, medulla, PMC, and cerebellum at all gestational and postnatal ages examined (Fig. 4*A*). The highest AP content occurred in late gestation (126 d or 137–138 d) for all regions except the medulla, where the highest content was observed at 90 d. In contrast to fetal content, AP was markedly lower at both 3 and 19–26 d of postnatal age in all regions examined. Progesterone content was low to undetectable in all brain regions in fetuses at 72 d gestation (Fig. 4*B*). The highest content was observed in the fetal brain at either 113 or 126 d gestation and had decreased significantly by 144 d gestation, before markedly decreasing further after birth. Pregnenolone was detected in all brain regions from 72 d gestation, and peak content was found at 113 d gestation with a gradual but significant decrease from this peak by 144 d gestation (Fig. 4*C*). There was a further significant decrease of pregnenolone content after birth to very low to undetectable levels in all brain regions in lambs of 19–26 d at postnatal age.

Adrenal Steroid Content

Adrenal AP, progesterone, and pregnenolone content increased with advancing gestation (Fig. 5). AP content was highest at 144 d gestation and then decreased significantly after birth (Fig. 5*A*). Progesterone content increased progressively from 90 d to 140 d gestation; these levels were maintained until at least 3 d after birth and then decreased significantly by 19–26 d of age (Fig. 5*B*). Pregnenolone content also increased progressively from 90 d gestation, until at least 3 d of postnatal age, followed by a significant decrease in the 19- to 26-d-old neonate (Fig. 5*C*).

Plasma Steroid Content

AP, progesterone, and pregnenolone were detectable in plasma at all fetal and neonatal ages examined (Fig. 6). The highest AP content was observed in fetal plasma between 113 and 137–138 d gestation (Fig. 6*A*), with content falling progressively after birth. Progesterone content increased progressively from 72 d gestation and was highest at 137–138 d gestation (Fig. 6*B*), before decreasing significantly by late gestation (144 d) and decreasing further after birth until reaching the limit of detection (1.2 ± 0.2 nmol/L) in the 19- to 26-d-old neonate. Pregnenolone content increased progressively with gestation until at least 144 d gestation (Fig. 6*C*) and

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited

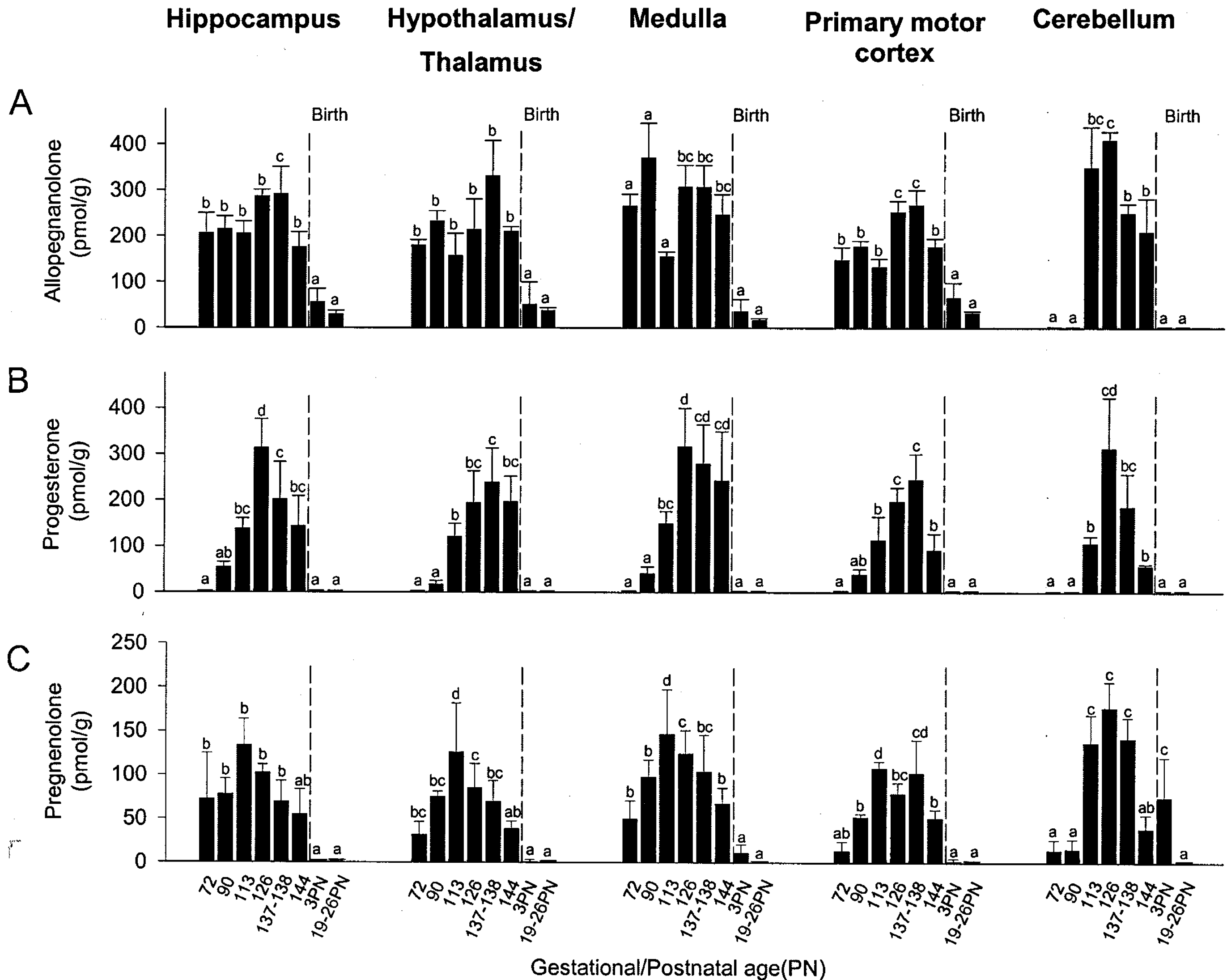

Figure 4. Content of AP (*A*), progesterone (*B*), and pregnenolone (*C*) in the hippocampus, hypothalamus/thalamus, medulla, PMC, and cerebellum measured in the brain of fetuses between 72 and 144 d gestation and lambs at 3 and 19–26 d of postnatal age. AP, progesterone, and pregnenolone content in late-gestation fetal brain were significantly greater than in the neonatal brain. Each bar represents the mean ± SEM for four animals. Different lower case lettering indicates significance difference, $p < 0.05$.

had decreased markedly by 3 d after birth; at 19–26 d of postnatal age, content was significantly lower than fetal values.

DISCUSSION

The principal finding of this study was that two key steroidogenic enzymes, P450scc and 5αRII, were strongly expressed in the hippocampus, hypothalamus/thalamus, medulla, PMC, and cerebellum of both the fetal and neonatal brain and also in the fetal adrenals. The overall expression of both enzymes was low before 90 d gestation and increased toward term, and in most regions, expression of the enzymes was then either maintained or increased further in postnatal life. Second, AP content in the brain, which was consistently high throughout late gestation, decreased profoundly at birth despite the high and sometimes increased levels of expression of the two steroidogenic enzymes in the postnatal brain.

We measured the expression of P450scc to determine whether pregnenolone, the obligate precursor for neuroactive steroid production, could be produced in the fetal brain. The finding that this enzyme was strongly expressed prenatally suggests that *de novo* pregnenolone production occurs *in utero* in the brain of this long gestation species. Although we are not able to partition the brain content of pregnenolone between peripheral and brain sites of production, the profound decrease of pregnenolone in the brain and plasma after birth suggests that the placenta may make a significant contribution to brain pregnenolone levels in fetal life. Alternatively, there may be a significant decrease in the mobilization of cholesterol at birth or that removal of the placenta and the intrauterine environment reduces another unidentified factor that induces pregnenolone production in the fetus.

Immunoreactive P450scc has been observed in the rat CNS from embryonic day 9.5 continuing throughout prenatal development (12), with the primary site of pregnenolone synthesis occurring in oligodendrocytes and astrocytes and with lower levels in neurons. P450scc is strongly expressed in the white

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited

matter (34), and neuronal expression of P450scc is generally lower than that of glia (13–16). Interestingly, the cerebellar Purkinje cell possesses this steroidogenic enzyme and produces neurosteroids *de novo* in a number of vertebrates, including mammalian species (20, 35, 36), particularly fetal and neonatal sheep (20). This is consistent with the high level of cerebellar P450scc expression found in the present study. The relative differences in the expression of P450scc and 5αRII between the different areas of the brain at a particular age may be attributed to differences in glia/neuron composition, and changes with age may be attributed to the different rates of proliferation and maturation of glia in particular areas of the brain. The finding that 5αRII is strongly expressed in the fetal brain suggests that *de novo* production of 5α-reduced progesterone metabolites is possible in the brain of fetal sheep.

The disparity between plasma and brain progesterone concentration and AP content before 126 d gestation further supports '*de novo*' synthesis in the brain. Progesterone produced in the brain may be rapidly converted to AP, accounting for the lower brain progesterone content at this time. Our findings are consistent with studies in the rat, which showed that AP content in the brain does not decline in parallel with the prepartum fall in plasma progesterone concentrations (37). Alternatively, the marked decline in AP after birth, despite the continued strong expression of P450scc and 5αRII, suggests that a minimal supply of peripheral precursors is required to maintain the high AP content in the fetal brain. The ovine placenta contains abundant P450scc and 3β-hydroxysteroid dehydrogenase and produces large amounts of progesterone, some of which is converted to 3α-hydroxyprogesterone and 20α-hydroxyprogesterone on reaching the fetal circulation (38). These metabolites may function as precursors for conversion to AP by 5αRII in the fetal brain. However, the brain also contains high levels of 3α-hydroxysteroid oxidoreductases (39), which are required for the conversion of 5α-reduced progesterone metabolites to AP. Therefore, the provision of 3α-reduced metabolites may not be essential for AP production. Although 3α-hydroxysteroid oxidoreductase is required

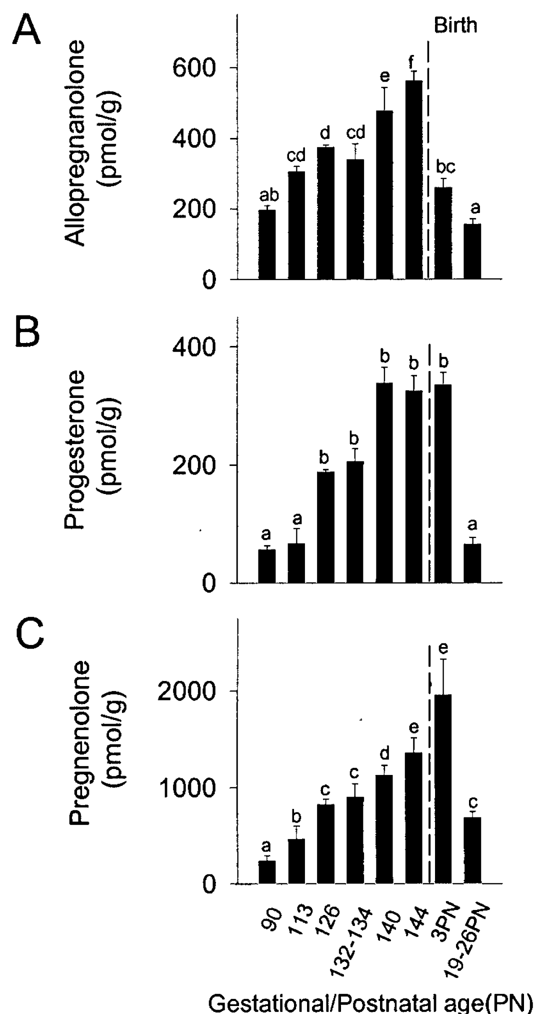

Figure 5. Content of AP (*A*), progesterone (*B*), and pregnenolone (*C*) in adrenal glands of fetuses between 72 and 144 d gestation and lambs at 3 and 19–26 d of postnatal age. Each bar represents the mean ± SEM for four animals. Different lower case lettering indicates significance difference, $p < 0.05$.

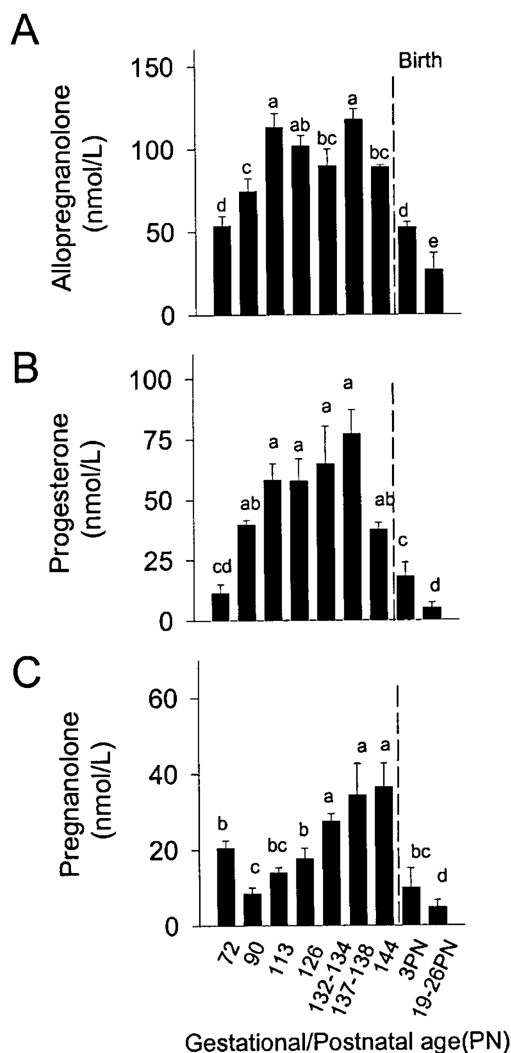

Figure 6. Plasma AP (*A*), progesterone (*B*), and pregnenolone (*C*) of fetuses between 72 and 144 d gestation and lambs at 3 and 19–26 d of postnatal age. Each bar represents the mean ± SEM for four animals. Different lower case lettering indicates significance difference, $p < 0.05$.

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited.

to produce AP, P450scc is known to be the slowest rate-limiting (V_{max} and K_m) steroidogenic enzyme (12) and 5αRII is considerably slower (V_{max}) than 3α-hydroxysteroid oxidoreductase (40, 41). Kokate *et al.* (42) showed that the regional distribution of 5α-DHP and AP are similar throughout the rat brain and differ markedly from the distribution of progesterone. These findings suggest that the 5α-reduction step may be critical in determining the quantity of 5α-reduced progesterone metabolites either produced from progesterone within the fetal brain or derived from precursors entering the brain from the blood.

Two isoforms of 5α-reductase have been identified, type I and type II, encoded by separate genes. 5αRI enzyme gene expression is constitutively expressed in the rat CNS at all stages of brain development, and expression is similar in male and female rats (21). The regulation of the 5αRII gene is different and is transiently expressed in the rat brain in late fetal/early postnatal life, and it has a 10- to 20-fold higher affinity for progesterone than the type I isoform (41). In the adult, expression is increased acutely after stress (21) but otherwise remains low and restricted to a few regions such as the hypothalamus. The action of 5αRII may be crucial for the local intracerebral formation of active anxiolytic/anesthetic steroids, derived from progesterone and some corticoids. In late gestation and at birth, when the 5αRII isoform is highly expressed, the high plasma levels of progesterone and its metabolites may be the substrate for the production of 5α-reduced, 3α-hydroxylated anesthetic compounds, such as AP. Thus, high maximal progesterone content and maximal 5αRII expression could be responsible for attenuating the stress of parturition in newborn animals (21). It is known that in the adult, sex differences can affect neurosteroid content; however, previous findings in the rat suggest that sex differentiation of brain neurosteroid levels does not occur until after 25 d postnatal age (43). Thus, it is likely that neurosteroids have similar roles in the male and female perinatal brain.

We have shown that $GABA_A$ receptor concentrations, equivalent to those in the adult brain, are present in the fetus (22). Importantly, the receptors in the fetal brain were more sensitive to AP, compared with the receptors in the adult brain (22). Our previous studies have also demonstrated the sedative effects of systemically administered pregnenolone and progesterone (44, 45) and an increase of fetal wakefulness produced by treatment with finasteride, a potent 5αRII inhibitor (7). The brain content of AP observed in the present study would be sufficient to modulate fetal $GABA_A$ receptors and increase the activity of GABAergic pathways in the fetal brain (22). Neurosteroids, including AP, also have neuroprotective functions (8), particularly in the hippocampal region. AP administration exerts a potent neuroprotective effects in cell viability studies in rat hippocampus slices and in primary hippocampal cultures as the steroid attenuates glutamate-induced increases of intracellular calcium (46). Thus, high levels of AP may protect the fetal brain; for example, neurosteroids that protect neurons after hypoxic or asphyxic episodes could be beneficial to the developing fetus.

We conclude that 5αRII and P450scc are expressed in the ovine fetal brain throughout the second half of gestation, and if the expressed protein leads to the formation of active enzyme, then this may allow the synthesis of 5α-pregnane steroids in the fetal brain. The decrease of AP at birth suggests that the placenta or another intrauterine factor has a critical role in maintaining the high content of AP in the brain, prenatally. Given the differences between brain and plasma content, it is likely that the high content of AP in the fetal brain results from both placental supply of precursors and *in situ* production in the CNS. Overall, our findings suggest that the loss of the placenta at birth leads to the dramatic decline in AP content in the brain of the newborn. This may remove a sedation-like effect from brain activity but may also leave the neonatal brain more vulnerable to excitotoxic injury induced by adverse events in the postpartum period.

REFERENCES

1. Crawley JN, Glowa JR, Majewska MD, Paul SM 1986 Anxiolytic activity of an endogenous adrenal steroid. Brain Res 398:382–385
2. Wieland S, Lan NC, Mirasedeghi S, Gee KW 1991 Anxiolytic activity of the progesterone metabolite 5 alpha-pregnan-3 alpha-o1-20-one. Brain Res 565:263–268
3. Belelli D, Bolger MB, Gee KW 1989 Anticonvulsant profile of the progesterone metabolite 5 alpha-pregnan-3 alpha-ol-20-one. Eur J Pharmacol 166:325–329
4. Devaud LL, Purdy RH, Morrow AL 1995 The neurosteroid, 3 alpha-hydroxy-5 alpha-pregnan-20-one, protects against bicuculline-induced seizures during ethanol withdrawal in rats. Alcohol Clin Exp Res 19:350–355
5. Celotti F, Melcangi RC, Martini L 1992 The 5 alpha-reductase in the brain: molecular aspects and relation to brain function. Front Neuroendocrinol 13:163–215
6. Nicol MB, Hirst JJ, Walker D, Thorburn GD 1997 Effect of alteration of maternal plasma progesterone concentrations on fetal behavioural state during late gestation. J Endocrinol 152:379–386
7. Nicol MB, Hirst JJ, Walker DW 2001 Effect of finasteride on behavioural arousal and somatosensory evoked potentials in fetal sheep. Neurosci Lett 306:13–16
8. Majewska MD, Harrison NL, Schwartz RD, Barker JL, Paul SM 1986 Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. Science 232:1004–1007
9. Baulieu EE 1997 Neurosteroids: of the nervous system, by the nervous system, for the nervous system. Recent Prog Horm Res 52:1–32
10. Paul SM, Purdy RH 1992 Neuroactive steroids. FASEB J 6:2311–2322
11. Zwain IH, Yen SS 1999 Neurosteroidogenesis in astrocytes, oligodendrocytes, and neurons of cerebral cortex of rat brain. Endocrinology 140:3843–3852
12. Compagnone NA, Bulfone A, Rubenstein JL, Mellon SH 1995 Steroidogenic enzyme P450c17 is expressed in the embryonic central nervous system. Endocrinology 136:5212–5223
13. Hu ZY, Bourreau E, Jung-Testas I, Robel P, Baulieu EE 1987 Neurosteroids: oligodendrocyte mitochondria convert cholesterol to pregnenolone. Proc Natl Acad Sci U S A 84:8215–8219
14. Jung-Testas I, Hu ZY, Baulieu EE, Robel P 1989 Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. Endocrinology 125:2083–2091
15. Baulieu EE 1991 Neurosteroids: a new function in the brain. Biol Cell 71:3–10
16. Akwa Y, Young J, Kabbadj K, Sancho MJ, Zucman D, Vourc'h C, Jung-Testas I, Hu ZY, Le Goascogne C, Jo DH, et al 1991 Neurosteroids: biosynthesis, metabolism and function of pregnenolone and dehydroepiandrosterone in the brain. J Steroid Biochem Mol Biol 40:71–81
17. Wilson JD, Griffin JE, Russell DW 1993 Steroid 5 alpha-reductase 2 deficiency. Endocr Rev 14:577–593
18. Russell DW, Wilson JD 1994 Steroid 5 alpha-reductase: two genes/two enzymes. Annu Rev Biochem 63:25–61
19. Melcangi RC, Celotti F, Martini L 1994 Progesterone 5-alpha-reduction in neuronal and in different types of glial cell cultures: type 1 and 2 astrocytes and oligodendrocytes. Brain Res 639:202–206
20. Petratos S, Hirst JJ, Mendis S, Anikijenko P, Walker DW 2000 Localization of P450scc and 5alpha-reductase type-2 in the cerebellum of fetal and newborn sheep. Brain Res Dev Brain Res 123:81–86
21. Poletti A, Coscarella A, Negri-Cesi P, Colciago A, Celotti F, Martini L 1998 5 alpha-reductase isozymes in the central nervous system. Steroids 63:246–251
22. Crossley KJ, Walker DW, Beart PM, Hirst JJ 2000 Characterisation of GABA(A) receptors in fetal, neonatal and adult ovine brain: region and age related changes and the effects of allopregnanolone. Neuropharmacology 39:1514–1522
23. Harlow E, Lane D 1988 Antibodies, A Laboratory Manual. Cold Spring Harbor Laboratory Press, New York
24. Okuyama E, Okazaki T, Furukawa A, Wu RF, Ichikawa Y 1996 Molecular cloning and nucleotide sequences of cDNA clones of sheep and goat adrenocortical cytochromes P450scc (CYP11A1). J Steroid Biochem Mol Biol 57:179–185

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited.

25. Andersson S, Russell DW 1990 Structural and biochemical properties of cloned and expressed human and rat steroid 5 alpha-reductases. Proc Natl Acad Sci U S A 87:3640–3644
26. Fields GB, Noble RL 1990 Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. Int J Pept Protein Res 35:161–214
27. Aumuller G, Eicheler W, Renneberg H, Adermann K, Vilja P, Forssmann WG 1996 Immunocytochemical evidence for differential subcellular localization of 5 alpha-reductase isoenzymes in human tissues. Acta Anat 156:241–252
28. Silver RI, Wiley EL, Thigpen AE, Guileyardo JM, McConnell JD, Russell DW 1994 Cell type specific expression of steroid 5 alpha-reductase 2. J Urol 152:438–442
29. Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
30. Barbaccia ML, Roscetti G, Trabucchi M, Ambrosio C, Massotti M 1992 Cyclic AMP-dependent increase of steroidogenesis in brain cortical minces. Eur J Pharmacol 219:485–486
31. Billiards SS, Walker DW, Canny BJ, Hirst JJ 2002 Endotoxin increases sleep and brain allopregnanolone concentrations in newborn lambs. Pediatr Res 52:892–899
32. Bernardi F, Salvestroni C, Casarosa E, Nappi RE, Lanzone A, Luisi S, Purdy RH, Petraglia F, Genazzani AR 1998 Aging is associated with changes in allopregnanolone concentrations in brain, endocrine glands and serum in male rats. Eur J Endocrinol 138:316–321
33. Rice GE, Thorburn GD 1986 Characterization of particle-associated choriomammotrophin and progesterone in ovine placentomes. J Endocrinol 111:217–223
34. Le Goascogne C, Robel P, Gouezou M, Sananes N, Baulieu EE, Waterman M 1987 Neurosteroids: cytochrome P-450scc in rat brain. Science 237:1212–1215
35. Takase M, Ukena K, Yamazaki T, Kominami S, Tsutsui K 1999 Pregnenolone, pregnenolone sulfate, and cytochrome P450 side-chain cleavage enzyme in the amphibian brain and their seasonal changes. Endocrinology 140:1936–1944
36. Tsutsui K, Ukena K, Takase M, Kohchi C, Lea RW 1999 Neurosteroid biosynthesis in vertebrate brains. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 124:121–129
37. Pomata PE, Colman-Lerner AA, Baranao JL, Fiszman ML 2000 In vivo evidences of early neurosteroid synthesis in the developing rat central nervous system and placenta. Brain Res Dev Brain Res 120:83–86
38. Seamark RF, Nancarrow CD, Gardiner J 1970 Progesterone metabolism in ovine blood: the formation of 3 alpha-hydroxy-pregn-4-en-20-one and other substances. Steroids 15:589–604
39. Li X, Bertics PJ, Karavolas HJ 1997 Regional distribution of cytosolic and particulate 5alpha-dihydroprogesterone 3alpha-hydroxysteroid oxidoreductases in female rat brain. J Steroid Biochem Mol Biol 60:311–318
40. Chetyrkin SV, Belyaeva OV, Gough WH, Kedishvili NY 2001 Characterization of a novel type of human microsomal 3alpha-hydroxysteroid dehydrogenase: unique tissue distribution and catalytic properties. J Biol Chem 276:22278–22286
41. Normington K, Russell DW 1992 Tissue distribution and kinetic characteristics of rat steroid 5 alpha-reductase isozymes. Evidence for distinct physiological functions. J Biol Chem 267:19548–19554
42. Bixo M, Andersson A, Winblad B, Purdy RH, Backstrom T 1997 Progesterone, 5alpha-pregnane-3,20-dione and 3alpha-hydroxy-5alpha-pregnane-20-one in specific regions of the human female brain in different endocrine states. Brain Res 764:173–178
43. Grobin AC, Morrow AL 2001 3Alpha-hydroxy-5alpha-pregnan-20-one levels and GABA(A) receptor-mediated 36Cl(-) flux across development in rat cerebral cortex. Brain Res Dev Brain Res 131:31–39
44. Nicol MB, Hirst JJ, Walker DW 1998 Effect of pregnane steroids on electrocortical activity and somatosensory evoked potentials in fetal sheep. Neurosci Lett 253:111–114
45. Nicol MB, Hirst JJ, Walker D 1999 Effects of pregnanolone on behavioural parameters and the responses to GABA(A) receptor antagonists in the late gestation fetal sheep. Neuropharmacology 38:49–63
46. Frank C, Sagratella S 2000 Neuroprotective effects of allopregnenolone on hippocampal irreversible neurotoxicity in vitro. Prog Neuropsychopharmacol Biol Psychiatry 24:1117–1126

Copyright © by International Pediatric Research Foundation, Inc. Unauthorized reproduction of this article is prohibited