0013-7227/99/$03.00/0
Endocrinology

Vol. 140, No. 4
Printed in U.S.A.

Pregnenolone, Pregnenolone Sulfate, and Cytochrome P450 Side-Chain Cleavage Enzyme in the Amphibian Brain and Their Seasonal Changes*

MINORU TAKASE, KAZUYOSHI UKENA, TAKESHI YAMAZAKI, SHIRO KOMINAMI, AND KAZUYOSHI TSUTSUI

Laboratory for Amphibian Biology, Faculty of Science (M.T.), and Laboratory of Brain Science, Faculty of Integrated Arts and Sciences (K.U., T.Y., S.K., K.T.), Hiroshima University, Higashi-Hiroshima 739-8521, Japan

ABSTRACT

To clarify whether the amphibian brain synthesizes *de novo* neurosteroids, we examined pregnenolone, pregnenolone sulfate ester, and cytochrome P450 side-chain cleavage enzyme (cytochrome P450scc), an enzyme converting cholesterol to pregnenolone, using amphibians. Pregnenolone and its sulfate ester in the brain, gonad, and plasma of *Xenopus laevis* were measured by a specific pregnenolone RIA. The concentrations of these two steroids in the female brain were significantly larger than those in the ovary and plasma. A similar tendency was evident in the male. In both sexes, pregnenolone and its sulfate ester were concentrated more highly in the cerebellum than in the telencephalon, diencephalon, or midbrain. An immunoreactive protein band of electrophoretic mobility in the proximity of bovine adrenal P450scc was detected in the *Xenopus* brain as well as the testis by Western blot analysis. Immunohistochemical analysis indicated that Purkinje cells in the *Xenopus* cerebellum were specifically immunostained with the P450scc antibody. P450scc-like immunoreactive cells were further found in several telencephalic and diencephalic regions, such as the pallium mediale and nucleus preopticus, in the *Xenopus* brain. A similar localization of P450scc-like immunoreactive cells was evident in *Rana nigromaculata*, a seasonally breeding amphibian. In the present study, seasonal changes in pregnenolone and its sulfate ester were further examined as a possible physiological change using *R. nigromaculata*. In both sexes, pregnenolone concentrations in the brain were almost constant during the seasonally breeding cycle. In contrast, the pregnenolone sulfate concentration in the brain was significantly lower in the hibernating quiescent phase and higher in the breeding and postbreeding active phases, independent of the plasma steroid level.

These results taken together suggest that the amphibian brain possesses steroidogenic enzyme P450scc and produces pregnenolone and its sulfate ester. Pregnenolone sulfate may function well during the breeding and postbreeding active phases of the year in the seasonal breeder. (*Endocrinology* **140:** 1936–1944, 1999)

PREGNENOLONE is the main precursor of various steroid hormones produced in peripheral steroidogenic glands and formed from cholesterol by the oxidative side-chain cleavage reaction, which is catalyzed by a specific enzyme, cytochrome P450 side-chain cleavage enzyme (cytochrome P450scc) (1). In addition to peripheral steroidogenic glands, it is now established that *de novo* steroidogenesis occurs in the mammalian brain from cholesterol. Pregnenolone and dehydroepiandrosterone, as unconjugated steroids, and their fatty acid or sulfate esters accumulate within the brain in several mammalian species, and the content of these steroids in the brain is almost constant even after the removal of peripheral steroids (2–8). Certain structures in the mammalian brain have the capacity to form pregnenolone (9–14). Recent studies further demonstrated that both P450scc protein and its messenger RNA are expressed in the rat brain (10, 11, 13, 15–18). Steroids synthesized in the nervous system are called neurosteroids (2). In mammals, glial cells are considered to play a major role in neurosteroid formation in the brain. P450scc has been found in the white matter throughout the rat brain (16). It has further been shown that both oligodendrocytes and astrocytes are the primary site for pregnenolone synthesis (9–13, 19). In addition to glial cells, P450scc was found in the rat Purkinje cell, a typical cerebellar neuron (18, 20).

On the other hand, we have recently demonstrated, using biochemical and immunochemical approaches, that the quail brain also possesses cytochrome P450scc and produces pregnenolone and its sulfate ester (21). In addition, our immunohistochemical studies with the same avian species have shown that P450scc-immunoreactive cells are distributed in several telencephalic, diencephalic, and mesencephalic regions (22, 23). In birds, both glial and neuronal cells also possess P450scc (22, 23).

In contrast to extensive studies with higher vertebrates, limited information has been available on neurosteroids in lower vertebrates. Recently, a series of studies using the amphibian brain (24–26) indicated that populations of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3βHSD)- or 17βHSD-immunoreactive cell bodies and fibers were observed in the telencephalon and diencephalon of the brain of *Rana ridibunda*. In addition, exogenous pregnenolone was metabolized to Δ^5-3β-hydroxysteroids and Δ^4-ketosteroids by brain slices of the same amphibian species (24–26) and the endozepine triakontatetraneuropeptide stimulated its

Received June 22, 1998.
Address all correspondence and requests for reprints to: Dr. Kazuyoshi Tsutsui, Laboratory of Brain Science, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan. E-mail: tsutsui@ipc.hiroshima-u.ac.jp.
* This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (08454265 and 10874129 to K.T.).

3βHSD activity (27). However, no study on pregnenolone biosynthesis from cholesterol, which is an initial step for steroidogenesis, has been reported to date in the amphibian brain.

With these findings as a background, we measured the intrabrain levels of pregnenolone and its sulfate ester and analyzed the presence of cytochrome P450scc in the brains of amphibians. The second purpose of this study was to determine seasonal changes in neurosteroids formed by P450scc as a possible physiological change using a seasonal breeder.

Materials and Methods

Animals

Adult male and female *Xenopus laevis* were purchased from the supplier of experimental animals (Wonderup Co., Ltd., Tokyo, Japan) and kept in dechlorinated tap water at 23 C. *Rana nigromaculata*, a seasonally breeding amphibian, was collected in its breeding phase. *X. laevis* and *R. nigromaculata* were kept in cages under natural fluctuations of ambient temperature and photoperiod and were given food and water *ad libitum* for a few days until termination by decapitation. To investigate seasonal changes in pregnenolone and its sulfate ester, *R. nigromaculata* was killed in April (breeding phase), September (postbreeding phase), and January (hibernating phase).

Preparation of plasma and tissue samples

To prepare plasma and brain tissue samples, amphibians were killed according to the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Hiroshima, Japan). The time lapse between the beginning and the end of the killing did not exceed 2 h, and this was always performed between 1000–1200 h. In *X. laevis*, trunk blood was collected into heparinized glass tubes and centrifuged at 1800 × *g* for 30 min at 4 C. The plasma samples were stored at −20 C. Immediately after blood collection, brains and gonads were removed and weighed. Some brains of *X. laevis* were carefully dissected into several regions under a microscope using a reference (28) of the frog brain as follows: telencephalon, diencephalon, midbrain, and cerebellum. The brain tissue and plasma samples were also collected in *R. nigromaculata*. All tissue samples were snap-frozen in liquid nitrogen and stored at −80 C.

Steroid extraction

Extraction of unconjugated steroids or their sulfates was performed according to the method reported previously (3, 18, 21). Individual frozen brain and gonads were rapidly thawed and separately homogenized in 5 ml cold PBS (0.14 M NaCl and 0.01 M potassium phosphate buffer, pH 7.6) with a Teflon-glass homogenizer. Individual plasma (500 μl) was diluted with 5 ml cold PBS. These tissue and plasma samples were applied to steroid extraction. To estimate the recovery of the unconjugated steroid during the extraction, 2000 cpm [7-^{3}H]pregnenolone were added to each tube with 5 ml ethyl acetate. The tubes were vigorously stirred for 30 min and centrifuged at 3000 × *g* for 5 min. The organic phase was removed, and the extraction step was repeated twice. The combined organic extracts, which contained unconjugated pregnenolone, were dried under a stream of nitrogen. For removal of steroids conjugated to fatty acid, the dried samples were dissolved in 2 ml 70% methanol by stirring for 5 min and were kept at −20 C overnight. After centrifugation at 3000 × *g* for 5 min, the supernatant was aspirated. The precipitate was washed with 2 ml cold 70% methanol by centrifugation at 3000 × *g* for 5 min, and the supernatant was aspirated. The combined supernatants were dried under a stream of nitrogen as the assay samples for unconjugated pregnenolone.

On the other hand, the steroid conjugated to sulfate was extracted from the remaining aquatic phase after extraction of ethyl acetate. For estimation of the recovery of the steroid sulfate ester during extraction, 2000 cpm [1,2,6,7-^{3}H]dehydroepiandrosterone sulfate ester were then added to each aquatic phase. The pH of the aquatic phase was decreased to 1 with 30 μl sulfuric acid, and saturated sodium chloride was added at a final concentration of 20%. Extraction of steroid sulfates in the aquatic phase with ethyl acetate was again performed as described above. The steroid sulfate was solvolized in 95% ethyl ether at 37 C overnight with shaking. The hydrolyzed steroid was washed once with 3 ml 1 N NaOH and twice with 3 ml distilled water and dried under a stream of nitrogen as the assay samples of pregnenolone sulfate ester. It might be possible to estimate the concentration of pregnenolone sulfate with RIA using an antipregnenolone serum, but the accuracy is low because of the low cross-reactivity (50%) to pregnenolone sulfate, as described below.

RIAs of pregnenolone and its sulfate ester

The dried residues were dissolved in 1 ml of PBS containing 0.1% gelatin. Each aqueous solution obtained from both extracts of organic and aquatic phases was divided into two aliquots: one aliquot for the recovery measurement, and the other for RIA of pregnenolone or its sulfate ester. To measure the concentrations of pregnenolone and its sulfate ester, aliquots from both extracts of organic and aquatic phases were applied to the specific RIA system of pregnenolone (3, 18, 21). In the RIA, [7-^{3}H]pregnenolone (SA, 23.5 Ci/mmol; New England Nuclear, Boston, MA) and the antiserum to pregnenolone (Radioassay Systems Laboratories, Inc., Immuchem Corp., Carson, CA) were used. The antiserum cross-reacted with pregnenolone sulfate at 50%, 17α-hydroxypregnenolone at 2%, and dehydroepiandrosterone at less than 0.01%, and the pregnenolone assay was performed without chromatographic purification of pregnenolone. Separation of bound and free steroids was performed by centrifugation after reaction with the IgG SORB (The Enzyme Center, Inc., Malden, MA) (18). The least detectable amount was 0.1 ng/ml, and interassay variation was less than 7%. The precision index of a linear portion of the competition curve, which was computed according to the method described previously (21, 29), was 0.037 in the assay. To verify the RIA system, competition curves were obtained using the tissue and plasma samples and were compared with a competition curve of pregnenolone.

Western immunoblot analysis with P450scc antibody

To detect cytochrome P450scc in the amphibian brain, Western immunoblot analysis was conducted after SDS-PAGE of tissue homogenates. The brain and testis of adult male *Xenopus* were rapidly thawed and separately homogenized in 4 vol ice-cold sample buffer containing 0.05% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM phenylmethylsulfonylfluoride. The homogenate was centrifuged at 15,000 × *g* for 20 min. The supernatant was concentrated by precipitation with 40% saturation of ammonium sulfate (18). Proteins derived from each tissue were subjected to SDS-10% PAGE, and then Western immunoblotting was performed according to our previous methods (18, 21). In brief, after transfer of electrophoresed proteins onto polyvinylidene difluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA), the blot was probed with a polyclonal antibody (1:1000 dilution) raised against the bovine adrenal cytochrome P450scc in the rabbit, followed by incubation with biotinylated goat antirabbit IgG (Vector Laboratories, Inc., Burlingame, CA) and finally by a 30-min incubation with avidin-biotin complex (Vectastain ABC Elite kit, Vector Laboratories, Inc.). The details of the characterization of this serum were reported previously (21, 23, 30). Immunoreactive bands were detected by immersing the membranes for 2–7 min in a diaminobenzidine (DAB) solution [0.05% DAB and 0.1% NiCl in 0.1 M phosphate buffer (PB), pH 7.4, containing 0.3% H_2O_2]. An amount of proteins was measured using the bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL) with BSA as a standard.

The specificity of the anti-P450scc serum was assessed by substitution of the control serum for the primary antiserum; in this control serum, the antibody (1:1000 dilution) was preabsorbed by incubation with the purified antigen in a saturating concentration (10 μg P450scc/ml) for 16 h before use. The membranes were incubated with this control serum, employing the same procedure as that for the anti-P450scc serum.

Immunohistochemical analysis with P450scc antibody

In the present immunohistochemical experiment, adult males of *X. laevis* and *R. nigromaculata* in the postbreeding phase (September 1998)

were deeply anesthetized with 10% 4-allyl-2-methoxyphenol (FA 100, Tanabe Pharmaceutical Co. Ltd., Osaka, Japan), and then perfused transcardially with 0.1 M PB followed by fixative solution (4% paraformaldehyde in 0.1 M PB). After dissection from the skull, brains were postfixed for 12 h in the same fixative solution at 4 C, and then soaked in a refrigerated sucrose solution (21% sucrose in 0.1 M PB) until they sank. Whole brains were embedded in OCT compound (Miles, Inc., Elkhart, IN) and frozen-sectioned sagittally at 8-μm thickness with a cryostat at −18 C. Every third section was grouped on a slide precoated with 3-aminopropyltriethoxysilane (Sigma Chemical Co., St. Louis, MO); thus, we obtained three independent series of adjacent sections. Only one of these series of sections was used for immunohistochemical staining with the anti-P450scc serum; the remaining two series were used for immunohistochemical staining with the antiserum (1:500 dilution) preincubated with a saturating concentration of the purified antigen (20 μg P450scc/ml) for 16 h before use as a control and for hematoxylin staining, respectively.

The sections were processed according to the routine avidin-biotin-peroxidase complex immunohistochemical technique (18, 23). Endogenous peroxidase activity was eliminated from the sections by incubation with 3% H_2O_2 in absolute methanol. After blocking nonspecific binding components with 5% normal goat serum and 1% BSA in PBS containing 0.3% Triton X-100, the sections were immersed with the anti-P450scc serum at a dilution of 1:500 for 36 h at 4 C. The primary immunoreaction was followed by a 60-min incubation with biotinylated antirabbit IgG (10 μg/ml; Vector Laboratories, Inc.) and finally by a 60-min incubation with avidin-biotin complex (Vectastain ABC Elite kit, Vector Laboratories, Inc.). Immunoreactive products were detected by immersing the sections for 5–10 min in a DAB solution (0.05% DAB in PBS containing 0.3% H_2O_2). The localization of immunoreactive cells in the amphibian brain was studied using an Olympus Corp. BH-2 microscope (New Hyde Park, NY).

Statistics

Statistics for linearity and parallelism of competition curves in the RIA were computed according to the method of Bliss (31). Results of the RIA were expressed as the mean ± SEM. Differences in the concentrations of pregnenolone and its sulfate ester were analyzed by Duncan's multiple range test or Mann-Whitney U test after verification of equality or inequality of variances among groups to be compared (31).

Results

Pregnenolone and its sulfate ester in the brain, gonad, and plasma

To verify the measurement of pregnenolone concentrations in the extracts employed to a RIA system in the present study, competition experiments were first performed using extracts of the brain and plasma of *X. laevis* as competitors. Each slope of their competition curves paralleled significantly ($P < 0.05$) a standard curve of unlabeled pregnenolone as a competitor. In the male *Xenopus*, pregnenolone concentrations in the brain (0.558 pmol/mg) tended to be higher than those in the testis (0.256 pmol/mg) and plasma (0.166 pmol/μl), although the differences were not statistically significant (Fig. 1, *left panel*). Pregnenolone sulfate concentrations in the brain (0.162 pmol/mg) were significantly ($P < 0.05$) higher than those in the testis (0.078 pmol/mg) and plasma (0.035 pmol/μl; Fig. 1, *right panel*). Also in the female *Xenopus*, both concentrations of pregnenolone and its sulfate ester in the brain (0.257 and 0.087 pmol/mg, respectively) were significantly ($P < 0.05$) higher than those in the ovaries (0.039 and 0.021 pmol/mg, respectively) and plasma (0.006 and 0.014 pmol/μl, respectively; Fig. 2).

To investigate the distribution of these two steroids in the *Xenopus* brain, we measured the concentrations of pregnenolone and its sulfate ester in the dissected telencephalon, diencephalon, midbrain, and cerebellum. In the male *Xenopus*, both pregnenolone and its sulfate ester concentrated more highly in the cerebellum (3.33 and 2.94 pmol/mg, respectively) than in the telencephalon (0.30 and 0.20 pmol/mg, respectively), diencephalon (0.57 and 0.65 pmol/mg, respectively), and midbrain (0.77 and 0.68 pmol/mg, respectively; Fig. 3). For these two steroids, the difference in pregnenolone concentrations between the cerebellum and other brain regions were statistically significant ($P < 0.05$). Also in the female *Xenopus*, both concentrations of pregnenolone and its sulfate ester in the cerebellum (4.51 and 2.44 pmol/mg, respectively) were significantly ($P < 0.05$) higher than those in the telencephalon (0.13 and 0.08 pmol/mg, respectively), diencephalon (1.13 and 0.12 pmol/mg, respectively), and midbrain (0.69 and 0.21 pmol/mg, respectively; Fig. 4).

Western immunoblot analysis of P450scc-like protein in the brain

To ascertain that pregnenolone accumulation is due to a conversion of cholesterol in the brain, we then investigated the presence of P450scc-like protein in the *Xenopus* brain by Western immunoblot analysis with the polyclonal antibody raised against bovine adrenal cytochrome P450scc. Several protein bands derived from the brain and testicular extracts reacted to the P450scc antibody in the male *Xenopus* (Fig. 5, *left panel*). Among multiple signals in the brain extract, a most intense band was detected, and its electrophoretic mobility was in the proximity of bovine adrenal P450scc (Fig. 5, *left panel*). The molecular mass of this immunoreactive protein was approximately 54 kDa judging from its electrophoretic mobility. The electrophoretic mobilities of other bands in the brain were much different from that of bovine adrenal P450scc (Fig. 5, *left panel*). Preabsorption of the antibody with an excess of purified P450scc antigen (10 μg/ml) failed to stain the 54-kDa protein band in these tissues (Fig. 5, *right panel*). The P450scc-like protein band was also detected in females, and the intensity of the band was similar in males and females (data not shown).

Localization of P450scc-like immunoreactive cells in the brain

Because the *Xenopus* brain contained a large amount of pregnenolone and pregnenolone sulfate ester (Figs. 1–4) and was suggested to possess P450scc-like protein (Fig. 5), we examined the localization of P450scc-like immunoreactive cells in the male *Xenopus* brain. Although P450scc-like immunoreactive cells were found in several telencephalic, diencephalic, and rhombencephalic regions, the striking observation was the distribution of stained cells in the cerebellar cortex. As shown in Fig. 6, an immunoreaction with the anti-P450 serum was restricted to large cell bodies lying at a zone between the molecular and granular layers. The distribution of immunoreactive cell bodies in the cerebellum was coincident with the location of somata of Purkinje cells, characterized by the immunohistochemical (Fig. 6, b and e) and hematoxylin stainings (Fig. 6, a and d). Furthermore, in their somata the most intense immunore-

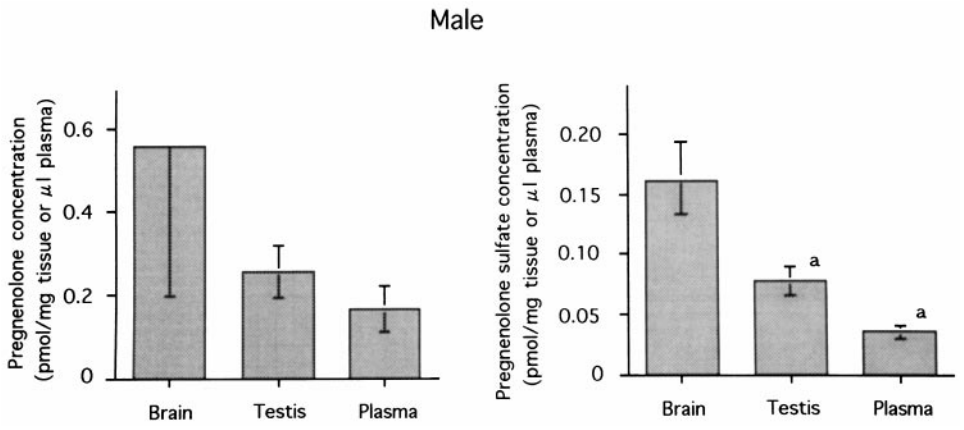

FIG. 1. Concentrations of pregnenolone (*left panel*) and pregnenolone sulfate (*right panel*) in the brain, testis, and plasma of adult male *X. laevis*. Each *column* and *vertical line* represent the mean ± SEM (n = 4 samples; one sample from one frog). Significance of difference: a, $P < 0.05$ *vs.* brain.

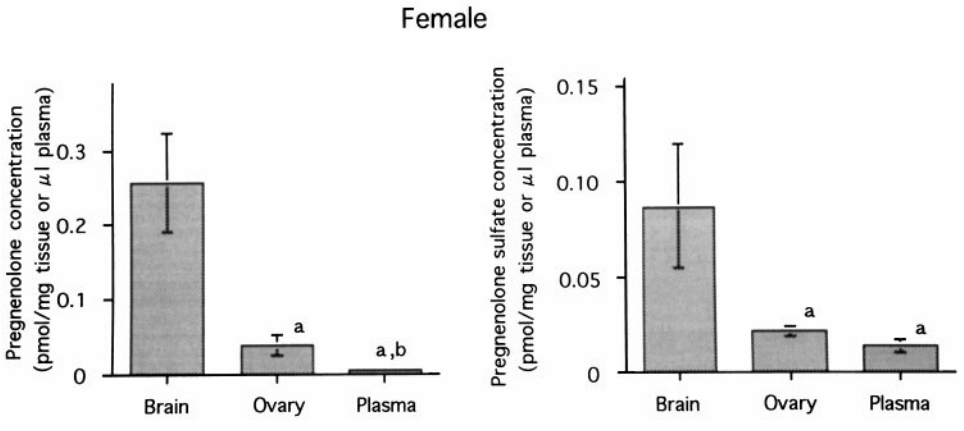

FIG. 2. Concentrations of pregnenolone (*left panel*) and pregnenolone sulfate (*right panel*) in the brain, ovary, and plasma of adult female *X. laevis*. Each *column* and *vertical line* represent the mean ± SEM (n = 4 samples; one sample from one frog). Significance of difference: a, $P < 0.05$ *vs.* brain; b, $P < 0.05$ *vs.* ovary.

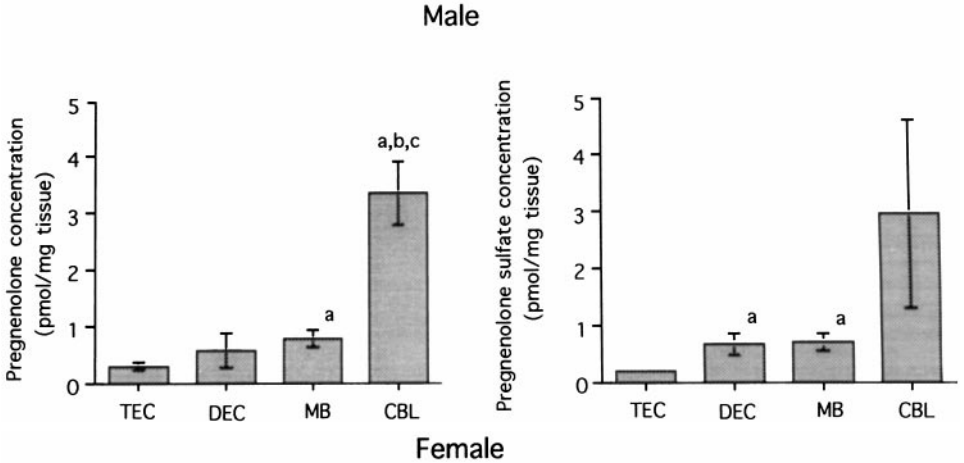

FIG. 3. Concentrations of pregnenolone (*left panel*) and pregnenolone sulfate (*right panel*) in different brain regions of adult male *X. laevis*. Brains were dissected into the telencephalon (TEC), diencephalon (DEC), midbrain (MB), and cerebellum (CBL). Each *column* and *vertical line* represent the mean ± SEM (n = 4 samples; one sample from one frog). Significance of difference: a, $P < 0.05$ *vs.* TEC; b, $P < 0.05$ *vs.* DEC; c, $P < 0.05$ *vs.* MB.

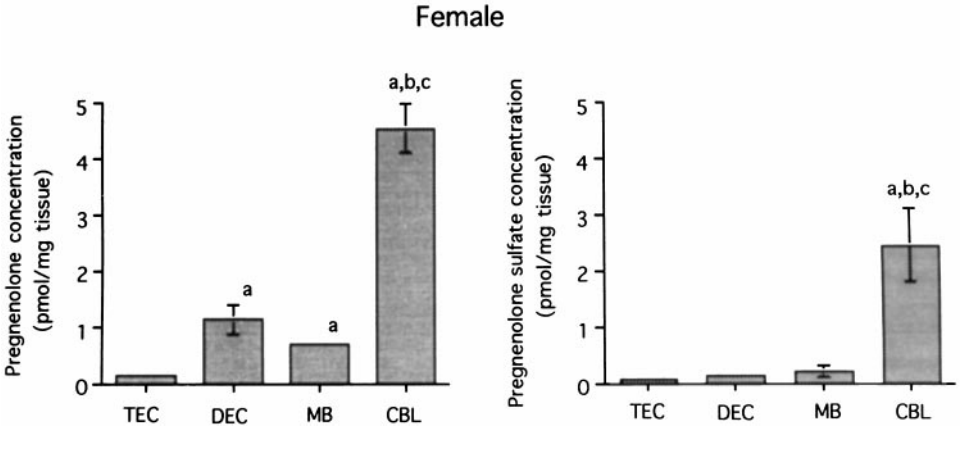

FIG. 4. Concentrations of pregnenolone (*left panel*) and pregnenolone sulfate (*right panel*) in different brain regions of adult female *X. laevis*. Brains were dissected into the telencephalon (TEC), diencephalon (DEC), midbrain (MB), and cerebellum (CBL). Each *column* and *vertical line* represent the mean ± SEM (n = 4 samples; one sample from one frog). Significance of difference: a, $P < 0.05$ *vs.* TEC; b, $P < 0.05$ *vs.* DEC; c, $P < 0.05$ *vs.* MB.

action was concentrated in a substantial number of granules, which suggests that some intracellular organelles, such as cytoplasmic mitochondria, may be the location of P450scc-like protein (Fig. 6e). Clusters of immunoreactive cell bodies were detected in the nucleus preopticus (Fig. 7a) and the nucleus infundibularis ventralis (Fig. 7b) in the diencephalic region. Some stained cells were also found in the pallium mediale (Fig. 7c) in the telencephalic regions. Preabsorption of the antibody with cytochrome P450scc resulted in no immunoreaction in the cerebellum (Fig. 6, c and f) or other brain regions (Fig. 7, d–f). Therefore, it is probable that the observed immunoreaction is due to the antibody recognizing endogenous cytochrome P450scc-like protein.

Immunohistochemical experiments conducted using the different amphibian species indicated similar results. Figure 8 shows immunoreactive cells located in the cerebellar Purkinje cell (a), the nucleus preopticus (b), and the nucleus infundibularis ventralis (c) in the diencephalic region of the male *R. nigromaculata* in the postbreeding phase (September).

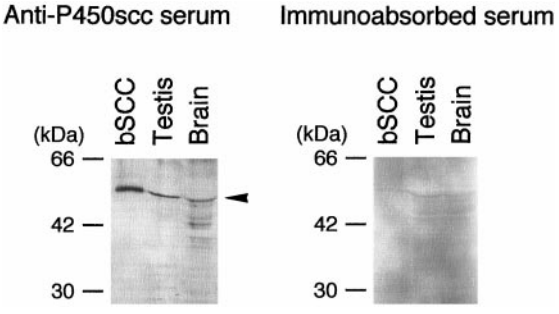

FIG. 5. Western immunoblot analysis of P450scc-like protein in the brain and testis of male *X. laevis*. Proteins of these tissues were treated with 2.5% SDS and electrophoresed on 10% polyacrylamide gels. Each lane contained 50 μg proteins from the respective tissues. Purified bovine adrenal P450scc (bSCC; 42 fmol) served as a reference marker. Western immunoblotting was performed as described in *Materials and Methods*. The blotted membranes were immunoreacted with the anti-P450scc serum (*left panel*) or with the antiserum preincubated with a saturating concentration of bSCC (*right panel*) as a control. The *arrowhead* indicates the P450scc-like protein band.

Seasonal changes in pregnenolone and its sulfate ester in the brain

In the final experiment, we examined seasonal changes in pregnenolone and its sulfate ester in the brain of *R. nigromaculata*, a seasonal breeder. The annual breeding cycle is divided into hibernating phase (November-March), the breeding phase (April-May), and the postbreeding phase (June-October). Pregnenolone concentrations in the male brain were almost constant throughout the three phases, whereas the plasma pregnenolone concentration was low and tended to increase during the postbreeding phase (September 1996; Fig. 9, *left panel*). In contrast, pregnenolone sulfate concentrations in the male brain were significantly ($P < 0.05$) higher during the postbreeding phase (September 1996) than during the hibernating phase (January 1997), whereas the plasma pregnenolone sulfate concentration was low and almost constant throughout the three phases (Fig. 9, *right panel*). Pregnenolone concentrations in the female brain were almost constant throughout the different phases (Fig. 10, *left panel*). Unlike males, the plasma pregnenolone concentration in females showed a similar level in the brain, and it was significantly ($P < 0.05$) higher during the hibernating (January 1997) and postbreeding (September 1996) phases than during the breeding phase (April 1996; Fig. 10, *left panel*). Pregnenolone sulfate concentrations in the female brain during the breeding phase (April 1996) were significantly ($P < 0.05$) higher than those during the hibernating phase (January 1997; Fig. 10, *right panel*). Similarly, the high level tended to remain during the postbreeding phase (September 1996; Fig. 10, *right panel*). In contrast to those in the brain, preg-

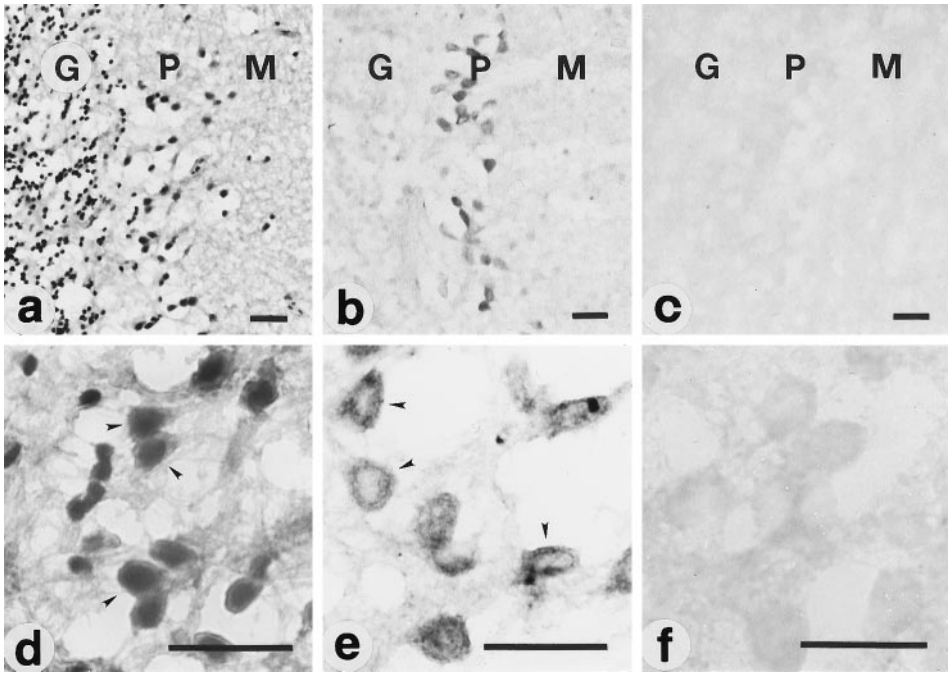

FIG. 6. Immunohistochemical staining with the antiserum to cytochrome P450scc (b and e) or with the antiserum preincubated with a saturating concentration of purified antigen (c and f) in the Purkinje cell layer (P) between the molecular (M) and granular (G) layers in the cerebellum of adult male *X. laevis*. Histology of the cerebellum was shown by hematoxylin staining (a and d). a, b, and c are of the same low magnification, and d, e, and f are of the same high magnification. The *arrowheads* indicate Purkinje cells (d and e). *Bars*, 30 μm. Immunohistochemical experiments were repeated independently four times using different frogs and produced similar results.

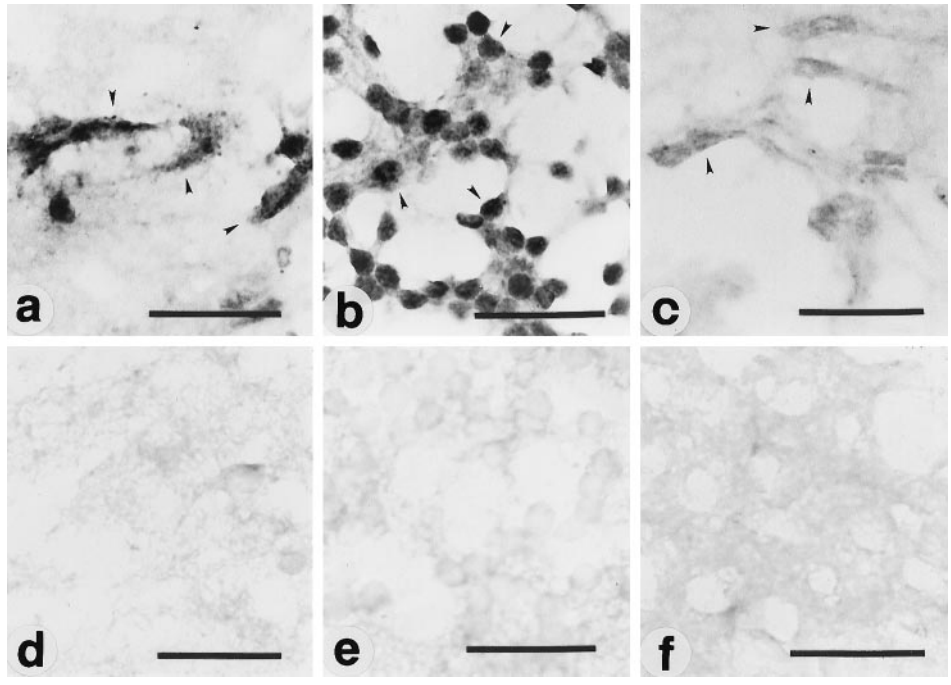

FIG. 7. Immunohistochemical staining with the antiserum to cytochrome P450scc (a–c) or with the antiserum preincubated with a saturating concentration of purified antigen (d–f) in diencephalic (a, b, d, and e) and telencephalic (c and f) regions of adult male *X. laevis*. a and d, The nucleus preopticus in the diencephalon; b and e, the nucleus infundibularis ventralis in the diencephalon; c and f, the pallium mediale in the telencephalon. The *arrowheads* indicate immunoreactive cells (a–c). *Bars*, 30 μm. Immunohistochemical experiments were repeated independently four times using different frogs and produced similar results.

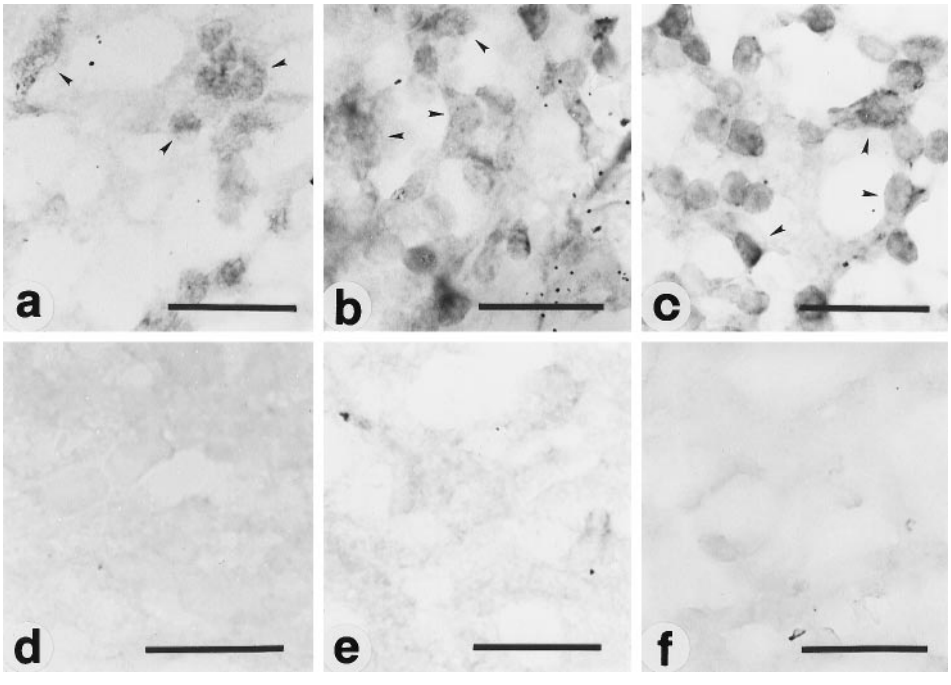

FIG. 8. Immunohistochemical staining with the antiserum to cytochrome P450scc (a–c) or with the antiserum preincubated with a saturating concentration of purified antigen (d–f) of adult male *R. nigromaculata* in the postbreeding phase (September). a and d, Purkinje cells in the cerebellar cortex; b and e, the nucleus preopticus in the diencephalon; c and f, the nucleus infundibularis ventralis in the diencephalon. The *arrowheads* indicate immunoreactive cells (a–c). *Bars*, 30 μm. Immunohistochemical experiments were repeated independently four times using different frogs and produced similar results.

nenolone sulfate concentrations in the female plasma were low and significantly ($P < 0.05$) increased during the postbreeding phase (September 1996; Fig. 10, *right panel*).

Discussion

In the present study we detected higher concentrations of pregnenolone and its sulfate ester in the brain of *X. laevis* (male, 0.558 and 0.162 pmol/mg tissue, respectively; female, 0.257 and 0.087 pmol/mg tissue, respectively) than in the gonad and plasma. Intrabrain concentrations of pregnenolone and its sulfate ester have also been measured in the anterior (~0.12 and ~0.038 pmol/mg tissue, respectively) and posterior (~0.07 and ~0.014 pmol/mg tissue, respectively) brains of adult male rats (3) and the brain of adult male Japanese quails (~0.19 and ~0.0036 pmol/mg tissue, respectively) (21) by specific RIAs. Thus, concentrations of these neurosteroids in the *Xenopus* brain may be higher than those in mammalian and avian brains. The present RIA results suggest that the amphibian brain possesses cytochrome P450scc, although we could not exclude the possibility that pregnenolone and/or its sulfate produced in the peripheral steroidogenic glands accumulate in the brain. Western im-

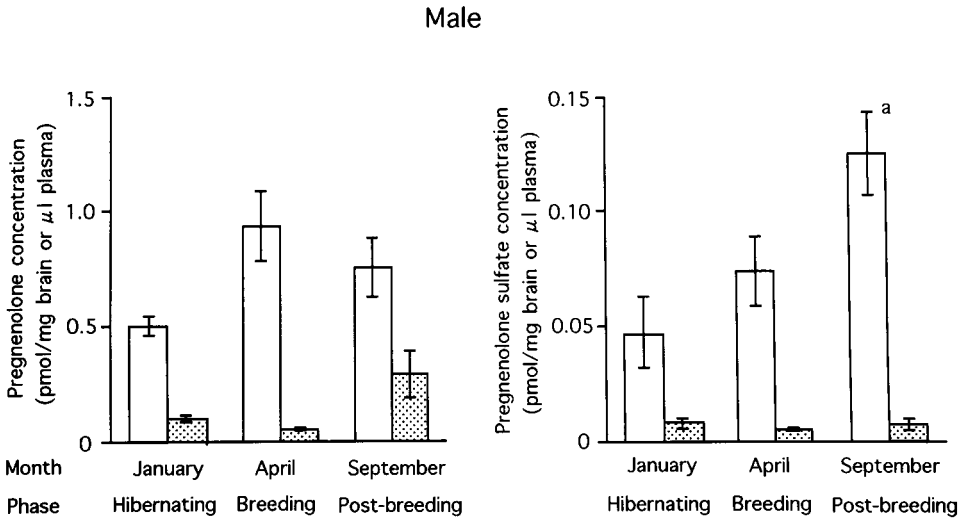

FIG. 9. Seasonal changes in concentrations of pregnenolone (*left panel*) and pregnenolone sulfate (*right panel*) in the brain (*open column*) and plasma (*dotted column*) from adult male *R. nigromaculata*. Frogs were killed in April (breeding phase), September (postbreeding phase), and January (hibernating phase). Each *column* and *vertical line* represent the mean ± SEM (n = 5 samples in each phase; one sample from one frog). Significance of difference: a, $P < 0.05$ *vs.* January.

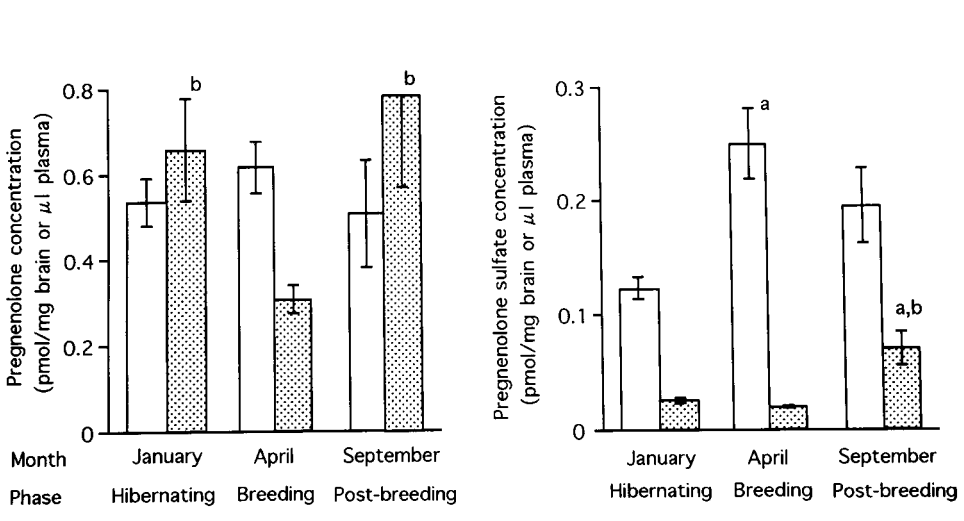

FIG. 10. Seasonal changes in concentrations of pregnenolone (*left panel*) and pregnenolone sulfate (*right panel*) in the brain (*open column*) and plasma (*dotted column*) from adult female *R. nigromaculata*. Frogs were killed in April (breeding phase), September (postbreeding phase), and January (hibernating phase). Each *column* and *vertical line* represent the mean ± SEM (n = 5 samples in each phase; one sample from one frog). Significance of difference: a, $P < 0.05$ *vs.* January; b, $P < 0.05$ *vs.* April.

munoblot analysis with the antibody against the purified bovine P450scc also suggests the presence of P450scc-like protein in the *Xenopus* brain, because only preabsorption of the antibody with an excess of purified P450scc antigen resulted in the complete disappearance of reaction product. In the *Xenopus* brain, the antibody predominantly recognized a protein band of electrophoretic mobility in the proximity of bovine P450scc as well as in the gonad. Other protein bands showing different electrophoretic mobilities were also detected in the brain. As the presence of P450scc in the brain has been established in several mammalian species (10, 11, 13, 15–18) and in an avian species (21–23), the presence of P450scc may be a conserved property of the vertebrate brain. However, to draw a firm conclusion, the biochemical demonstration of pregnenolone biosynthesis in the brain is needed in the amphibian species and other lower vertebrates.

The *Xenopus* cerebellum contained much higher concentrations of pregnenolone and its sulfate ester than did the telencephalon, diencephalon, and midbrain. The present study further identified the presence of P450scc-like protein in the cerebellar Purkinje cell by immunohistochemical analysis. This immunohistochemical finding is in agreement with our previous findings obtained in the mammalian (18, 20) and avian (22, 23) species. Interestingly, Netchitailo *et al.* (32) reported that atrial natriuretic factor (ANF)-like immunoreactive fibers innervate the Purkinje cell layer of the frog *Rana ridibunda*. In addition, ANF may contribute to the regulation of adrenal steroidogenesis in amphibians (33). It may be therefore possible that ANF is involved in the control of P450scc-containing Purkinje cells in amphibians. On the other hand, the presence of 3βHSD and 17βHSD in the brain of *R. ridibunda* has previously been reported (24–26). However, 3βHSD- and 17βHSD-immunoreactive cells were detected in the telencephalon and diencephalon, unlike in the cerebellum of *R. ridibunda* (24–26). In addition, the *Rana* rhombencephalon failed to metabolize pregnenolone to 17α-hydroxypregnenolone, suggesting the absence of 17α-hydroxylase/$C_{17,20}$-lyase (cytochrome P450c17) in the amphibian cerebellum (24–26). Therefore, it may be considered that pregnenolone and its sulfate ester contribute as major neurosteroids to some cerebellar function in the amphib-

ian brain. To identify the corresponding steroid products in the cerebellum as well as other brain regions, more precise experiments are required in amphibians.

If pregnenolone and its sulfate ester produced in the amphibian brain are involved in some physiological actions in the brain, these neurosteroids would change under different physiological conditions. To test this hypothesis, therefore, we examined seasonal changes in the concentrations of pregnenolone and its sulfate ester in the brain of *R. nigromaculata*, a seasonally breeding amphibian. In both sexes, pregnenolone sulfate concentrations in the *Rana* brain were high during the active season, *i.e.* breeding phase (female) and postbreeding phase (male), and low during the quiescent season, *i.e.* hibernating phase, whereas pregnenolone concentrations were almost constant in the brain throughout the year. In addition, such a seasonal change in pregnenolone sulfate observed in the brain may be independent of peripheral steroidogenic glands, because the change in plasma pregnenolone sulfate was clearly different from that in the brain. Further experiments are needed to clarify the significance of different seasonal changes in pregnenolone and pregnenolone sulfate in the amphibian brain.

It has been reported that in the mammalian brain pregnenolone and its sulfate interact as neurosteroids with the γ-aminobutyric acid A ($GABA_A$) receptor (34–37). Modulatory effects of the neuroactive steroid pregnanolone on $GABA_A$ receptor have been also investigated in amphibian pituitary melanotrophs (38, 39). GABA is accepted as an inhibitory neurotransmitter, and pregnenolone sulfate is generally considered to act as an antagonist of GABAergic neurotransmission (35–37). Various regions of the amphibian brain, such as the olfactory bulb, telencephalic dorsal and medial pallium, diencephalon, and cerebellum, contained GABA-like immunoreactive neurons (40, 41). The present and previous results taken together suggest that a higher level of pregnenolone sulfate during the active season may act as a stimulatory modulator to increase neuronal activities in the amphibian brain. The amphibian quiescent phase might change to the active phase by the action of pregnenolone sulfate in the brain. However, we need to examine a season-specific change in this neurosteroid in restricted brain regions with both P450scc and steroid sulfokinase, such as the cerebellar cortex and some diencephalic areas.

References

1. **Lieberman S, Greenfield NJ, Wolfson A** 1984 A heuristic proposal for understanding steroidogenic processes. Endocr Rev 5:128–148
2. **Corpëchot C, Robel P, Axelson M, Sjövall J, Baulieu EE** 1981 Characterization and measurement of dehydroepiandrosterone sulfate in rat brain. Proc Natl Acad Sci USA 78:4704–4707
3. **Corpëchot C, Synguelakis M, Talha S, Axelson M, Sjövall J, Vihko R, Baulieu EE, Robel P** 1983 Pregnenolone and its sulfate ester in rat brain. Brain Res 270:119–125
4. **Jo DH, Abdallah MA, Young J, Baulieu EE, Robel P** 1989 Pregnenolone, dehydroepiandrosterone, and their sulfate and fatty acid esters in the rat brain. Steroids 54:287–297
5. **Lanthier A, Patwardhan VV** 1986 Sex steroids and 5-en-3β-hydroxysteroids in specific regions of the human brain and cranial nerves. J Steroid Biochem 25:445–449
6. **Robel P, Baulieu EE** 1985 Neuro-steroids, 3β-hydroxy-Δ5-derivatives in the rodent brain. Neurochem Int 7:953–958
7. **Robel P, Bourreau E, Corpëchot C, Dang DC, Halberg F, Clarke C, Haug M, Schlegel ML, Synguelakis M, Vourc'h C, Baulieu EE** 1987 Neuro-steroids: 3β-hydroxy-Δ5-derivatives in rat and monkey brain. J Steroid Biochem 27:649–655
8. **Robel P, Corpëchot C, Clarke C, Groyer A, Synguelakis M, Vourc'h C, Baulieu EE** 1986 Neuro-steroids: 3β-hydroxy-Δ5-derivatives in the rat brain. In: Fink G, Harmar AJ, McKerns KW (eds) Neuroendocrine Molecular Biology. Plenum Press, New York, pp 367–377
9. **Akwa Y, Young J, Kabbadj K, Sancho MJ, Zucman D,Vourc'h C, Jung-Testas I, Hu ZY, Le Goascogne C, Jo DH, Corpëchot C, Simon P, Baulieu EE, Robel P** 1990 Neurosteroids: biosynthesis, metabolism and function of pregnenolone and dehydroepiandrosterone in the brain. J Steroid Biochem Mol Biol 40:71–81
10. **Baulieu EE** 1991 Neurosteroids: a new function in the brain. Biol Cell 71:3–10
11. **Baulieu EE, Robel P** 1990 Neurosteroids: a new brain function? J Steroid Biochem Mol Biol 37:395–403
12. **Hu ZY, Bourreau E, Jung-Testas I, Robel P, Baulieu EE** 1987 Neurosteroids: oligodendrocyte mitochondria convert cholesterol to pregnenolone. Proc Natl Acad Sci USA 84:8215–8219
13. **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
14. **Robel P, Baulieu EE** 1994 Neurosteroids: biosynthesis and function. Trends Endocrinol Metab 5:1–8
15. **Compagnone NA, Bulfone A, Rubenstein JLR, Mellon SH** 1995 Expression of the steroidogenic enzyme P450scc in the central and peripheral nervous systems during rodent embryogenesis. Endocrinology 136:2689–2696
16. **Le Goascogne C, Robel P, Gouézou M, Sananes N, Baulieu EE, Waterman M** 1987 Neurosteroids: cytochrome P-450scc in rat brain. Science 237:1212–1215
17. **Mellon SH, Deschepper CF** 1993 Neurosteroid biosynthesis: genes for adrenal steroidogenic enzymes are expressed in the brain. Brain Res 629:283–292
18. **Ukena K, Usui M, Kohchi C, Tsutsui K** 1998 Cytochrome P450 side-chain cleavage enzyme in the cerebellar Purkinje neuron and its neonatal change in rats. Endocrinology 139:137–147
19. **Papadopoulos V, Guarneri P, Krueger KE, Guidotti A, Costa E** 1992 Pregnenolone biosynthesis in C6–2B glioma cell mitochondria: regulation by a mitochondrial diazepam binding inhibitor receptor. Proc Natl Acad Sci USA 89:5113–5117
20. **Ukena K, Kohchi C, Tsutsui K** 1999 Expression and activity of 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase in the rat Purkinje neuron during neonatal life. Endocrinology 140:805–813
21. **Tsutsui K, Yamazaki T** 1995 Avian neurosteroids. I. Pregnenolone biosynthesis in the quail brain. Brain Res 678:1–9
22. **Tsutsui K, Yamazaki T, Usui M, Furukawa Y, Ukena K, Kohchi C, Kominami S** 1997 P450scc activity in the brain. In: Etches R, Harvey S, Sharp PJ (eds) Perspectives in Avian Endocrinology. Journal of Endocrinology Ltd, Bristol, pp 427–436
23. **Usui M, Yamazaki T, Kominami S, Tsutsui K** 1995 Avian neurosteroids. II. Localization of a cytochrome P450scc-like substance in the quail brain. Brain Res 678:10–20
24. **Mensah-Nyagan AG, Do-Rego JL, Feuilloley M, Marcual A, Lange C, Pelletier G, Vaudry H** 1996 In vivo and in vitro evidence for the biosynthesis of testosterone in the telencephalon of the female frog. J Neurochem 67:413–422
25. **Mensah-Nyagan AG, Feuilloley M, Do-Rego JL, Marcual A, Lange C, Tonon MC, Pelletier G, Vaudry H** 1996 Localization of 17β-hydroxysteroid dehydrogenase and characterization of testosterone in the brain of the male frog. Proc Natl Acad Sci USA 93:1423–1428
26. **Mensah-Nyagan AG, Feuilloley M, Dupont E, Do-Rego JL, Leboulenger F, Pelletier G, Vaudry H** 1994 Immunocytochemical localization and biological activity of 3β-hydroxysteroid dehydrogenase in the central nervous system of the frog. J Neurosci 14:7306–7318
27. **Do-Rego JL, Mensah-Nyagan AG, Feuilloley M, Ferrara P, Pelletier G, Vaudry H** 1998 The endozepine triakontatetraneuropeptide diazepam-binding inhibitor [17–50] stimulates neurosteroid biosynthesis in the frog hypothalamus. Neuroscience 83:555–570
28. **Wada M, Urano A, Gorbman A** 1980 A stereotaxic atlas for diencephalic nuclei of the frog, *Rana pipiens*. Arch Histol Jpn 43:157–173
29. **Tsutsui K** 1991 Pituitary and gonadal hormone-dependent and -independent induction of follicle-stimulating hormone receptors in the developing testis. Endocrinology 128:477–487
30. **Ikushiro S, Kominami S, Takemori S** 1992 Adrenal P-450scc modulates activity of $P\text{-}450_{11\beta}$ in liposomal and mitochondrial membranes. J Biol Chem 267:1464–1469
31. **Bliss CI** 1952 The Statistics of Bioassay. Academic Press, New York
32. **Netchitailo P, Feuilloley M, Pelletier G, Leboulenger F, Cantin M, Gutkowska J, Vaudry H** 1987 Atrial natriuretic factor-like immunoreactivity in the central nervous system of the frog. Neuroscience 22:341–359
33. **Lihrmann I, Netchitaïlo P, Feuilloley M, Cantin M, Delarue C, Leboulenger F, De Léan A, Vaudry H** 1988 Effect of atrial natriuretic factor on corticosteroid production by perifused frog interrenal slices. Gen Comp Endocrinol 71:55–62
34. **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

35. **Majewska MD, Schwartz RD** 1987 Pregnenolone-sulfate: an endogenous antagonist of the γ-aminobutyric acid receptor complex in the brain? Brain Res 404:355–360
36. **Majewska MD, Mienville JM, Vicini S** 1988 Neurosteroid pregnenolone sulfate antagonizes electrophysiological responses to GABA in neurons. Neurosci Lett 90:279–284
37. **Mienville JM, Vicini S** 1989 Pregnenolone sulfate antagonizes $GABA_A$ receptor-mediated currents via a reduction of channel opening frequency. Brain Res 489:190–194
38. **Le Foll F, Castel H, Louiset E, Vaudry H, Cazin L** 1997 Multiple modulatory effects of the neuroactive steroid pregnanolone on $GABA_A$ receptor in frog pituitary melanotrophs. J Physiol 504:387–400
39. **Le Foll F, Louiset E, Castel H, Vaudry H, Cazin L** 1997 Electrophysiological effects of various neuroactive steroids on the $GABA_A$ receptor in pituitary melanotrope cells. Eur J Pharmacol 331:303–311
40. **Franzoni MF, Morino P** 1989 The distribution of GABA-like-immunoreactive neurons in the brain of the newt, *Triturus cristatus* carnifex, and the green frog, *Rana esculenta.* Cell Tissue Res 255:155–166
41. **Reichenberger I, Streit P, Ottersen OP, Dieringer N** 1993 GABA- and glycine-like immunoreactivities in the cerebellum of the frog. Neurosci Lett 154:89–92