

Localization of 17 β -hydroxysteroid dehydrogenase and characterization of testosterone in the brain of the male frog

(neurosteroids/immunocytochemistry/mass spectrometry/androgen biosynthesis/glial cells)

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ABSTRACT Several enzymes involved in the formation of steroids of the pregnene and pregnane series have been identified in the brain, but the biosynthesis of testosterone has never been reported in the central nervous system. In the present study, we have investigated the distribution and bioactivity of 17 β -hydroxysteroid dehydrogenase (17 β -HSD) (EC 1.1.1.62; a key enzyme that is required for the formation of testosterone and estradiol) in the brain of the male frog *Rana ridibunda*. By using an antiserum against human type I placental 17 β -HSD, immunoreactivity was localized in a discrete group of ependymal glial cells bordering the telencephalic ventricles. HPLC analysis of telencephalon and hypothalamus extracts combined with testosterone radioimmunoassay revealed the existence of two peaks coeluting with testosterone and 5 α -dihydrotestosterone. After HPLC purification, testosterone was identified by gas chromatography/mass spectrometry. Incubation of telencephalon slices with [³H]pregnenolone resulted in the formation of metabolites which coeluted with progesterone, 17 α -hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone, and 5 α -dihydrotestosterone. The newly synthesized steroid comigrating with testosterone was selectively immunodetected by using testosterone antibodies. These data indicate that 17 β -HSD is expressed in a subpopulation of gliocytes in the frog telencephalon and that telencephalic cells are capable of synthesizing various androgens, including dehydroepiandrosterone, androstenedione, testosterone, and 5 α -dihydrotestosterone.

The enzyme 17 β -hydroxysteroid dehydrogenase (17 β -HSD; EC 1.1.1.62) plays a pivotal role in the biosynthesis of sex steroids by catalyzing the conversion of androstenedione into testosterone and estrone into estradiol. The occurrence of 17 β -HSD activity has been demonstrated in steroid hormone-producing organs, such as testis (1), ovary (2), adrenal (3), and placenta (4), and in a number of other tissues, including liver, prostate, breast, lung, and skin (3). Molecular cloning has shown the existence of two distinct mRNAs encoding 17 β -HSD in the human placenta (5), suggesting that multiple variants of this enzyme may be expressed in various organs.

Since the initial demonstration of the formation of dehydroepiandrosterone (DHEA) sulfate in the rat brain (6), a number of studies have shown that biosynthesis of various steroid hormones occurs in the central nervous system (7–9). In particular, we have recently observed the formation of 17 α -hydroxyprogesterone (a precursor of androgens and estrogens) in the frog diencephalon, suggesting that the brain possesses the capability of synthesizing sex steroids (10).

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However, the presence of 17 β -HSD in the brain has not yet been directly demonstrated.

In the present study, we have investigated the distribution of 17 β -HSD-immunoreactive cells in the brain of the male frog *Rana ridibunda* by using an antiserum against human placental 17 β -HSD, and we have studied the ability of frog brain tissue to synthesize sex steroids.

MATERIALS AND METHODS

Animals. Adult male frogs (*R. ridibunda*) were maintained under artificial illumination (light on from 0600 to 1800) and were kept under running water for at least 1 week before being sacrificed. For surgical operations and fixation, animals were anesthetized by immersion in 0.1% 3-aminobenzoic acid ethyl ester (MS222; Sandoz Pharmaceutical). To limit possible variations due to circadian rhythms (11), all animals were killed between 0930 and 1030.

Immunofluorescence Procedure. Animals were anesthetized and then perfused transcardially with 50 ml of 0.1 M phosphate buffer (pH 7.3) followed by 50 ml of Bouin's fixative. The brains were postfixed for 24 h, embedded in Tissue Teck (Reichert–Jung), frozen at –80°C, and cut in the frontal or sagittal planes in a cryostat (7- μ m thick sections). The slices were processed for the indirect immunofluorescence technique with an antiserum raised against purified human type I placental 17 β -HSD (5). The specificity of the 17 β -HSD antiserum was verified by immunoblot analysis. It was shown that the antiserum could only detect one band corresponding to purified human type I 17 β -HSD (12, 13). Other steroidogenic enzymes, such as 3 β -hydroxysteroid dehydrogenase (3 β -HSD), sulfatase, and 5 α -reductase, were not recognized by the 17 β -HSD antiserum (12, 13). Consecutive sections were stained with the 17 β -HSD antiserum and an antiserum produced against a synthetic fragment of human diazepam binding inhibitor [DBI-(33–50)]. The preparations were examined under a Leitz Orthoplan microscope or a confocal laser scanning microscope (Leica).

Tissue Extraction. Frogs were decapitated, and the telencephalon, hypothalamus, and rhombencephalon were rapidly dissected. Blood was collected by intracardiac puncture. Tissue samples were homogenized in 2.5 ml of ice-cold 10% (wt/vol) trichloroacetic acid and submitted to three successive extractions by 2.5 ml of dichloromethane. The aqueous supernatant was used for determination of the protein content. The organic

Abbreviations: ODN, octadecaneuropeptide; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 isomerase; Δ^5 P, pregnenolone; 17OH- Δ^5 P, 17-hydroxypregnenolone; 17OH-P, 17-hydroxyprogesterone; DHEA, dehydroepiandrosterone; 5 α -DHT, 5 α -dihydrotestosterone; DBI, diazepam binding inhibitor.

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phases were pooled, and the solvent was evaporated under a stream of nitrogen. The dry extracts were prepurified on Sep-Pak C₁₈ cartridges (Waters). The solvent was evaporated under nitrogen, and the extracts were kept dry until HPLC analysis.

The recovery rates of exogenous testosterone were as follows: plasma, 74.2% ± 3.9%; telencephalon, 74.0% ± 4.0%; hypothalamus, 87.0% ± 6.0%; and rhombencephalon, 85.6% ± 3.6%. All testosterone concentrations reported in *Results* are corrected for recovery.

Pulse-Chase Technique. For each experiment, the telencephalons of four frogs were rapidly dissected and cut into two slices. The tissue slices were incubated at 24°C for 0.5–4 h in 500 μl of Ringer's medium containing 1 μM [³H]pregnenolone ([³H]Δ⁵P) and 4% (vol/vol) propylene glycol as described (10). At the end of the incubation period, the tissue slices were rinsed four times with ice-cold Ringer's buffer and the experiment was stopped by addition of 750 μl of trichloroacetic acid. Steroids were extracted and prepurified as described above.

HPLC. Endogenous steroids or radioactive steroids formed by conversion of [³H]Δ⁵P were analyzed by reversed-phase HPLC as described (10). For radioimmunoassay of endogenous testosterone or immunodetection of newly synthesized [³H]testosterone, the fractions were collected at 0.5-min intervals and evaporated in a Speed-Vac Concentrator (Savant). For measurement of radioactive metabolites of [³H]Δ⁵P, tritiated compounds eluted from the HPLC column were directly

monitored with a flow scintillation analyzer (Radiomatic Flo-One/Beta A-500; Packard) equipped with a 486DX50 personal computer.

Measurement of Endogenous Testosterone. The concentrations of testosterone in the blood, as well as in the telencephalon and hypothalamus, were determined by radioimmunoassay after HPLC analysis of the tissue extracts by using an antiserum which exhibited 60% cross-reactivity with 5α-dihydrotestosterone (5α-DHT) and 49% with 5β-DHT. The antiserum also cross-reacted with epitestosterone (3.5%), 5α-androstane-3α, 17β-diol (2%), and 5α-androstane-3β, 17β-diol (4%). Cross-reactivity with all other steroids tested, including progesterone, cortisol, and corticosterone, was less than 1% (14). The concentration of testosterone in each extract was calculated from the area under the peak measured in three independent chromatograms.

Gas Chromatography-Mass Spectrometry (GC-MS). Frog telencephalon extracts (from 300 frog brains each) were analyzed by HPLC, and a sample of the peak coeluting with testosterone (≈20 ng of endogenous testosterone) was characterized by GC-MS using a Jeol-AX-500 GC-MS apparatus equipped with a Chrompack capillary column (CP SIL 5CB; length, 25 m; i.d., 0.32 mm; film thickness, 1.2 μm). Synthetic testosterone (10 ng), analyzed under the same conditions, was used as a reference standard.

Immunodetection Technique. Pulse-chase fractions purified by HPLC were reconstituted in 200 μl of phosphate buffer

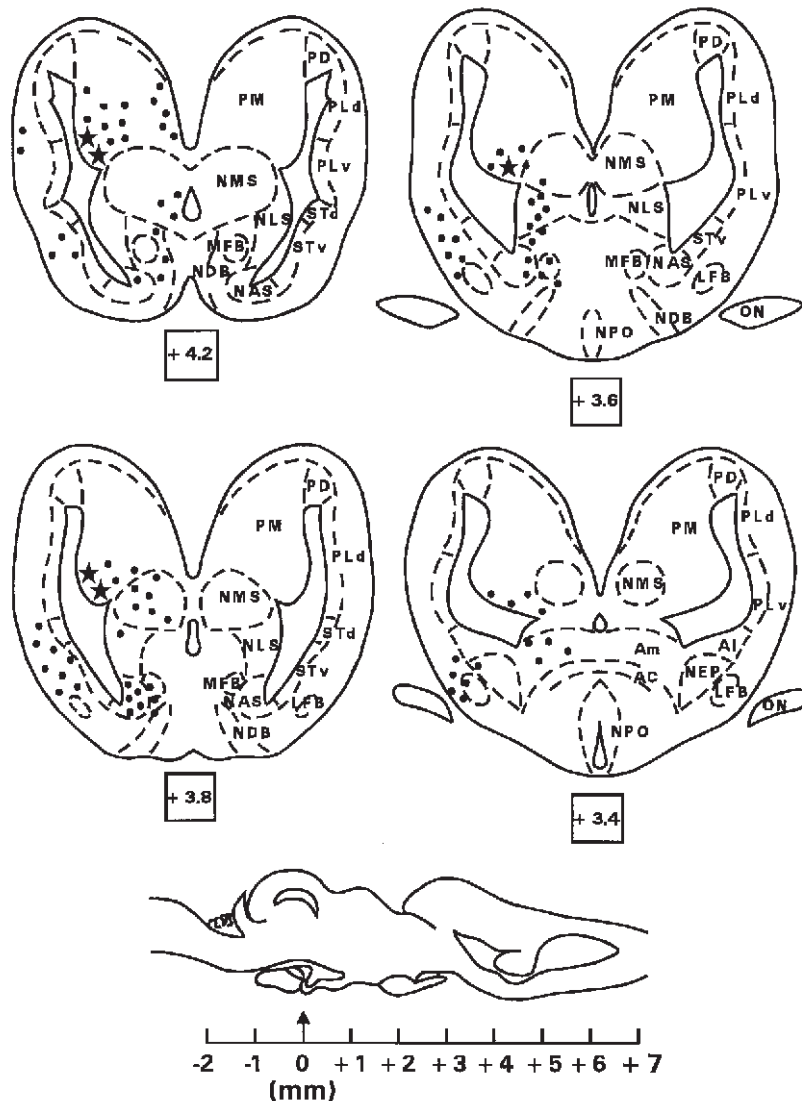


FIG. 1. Schematic frontal sections illustrating the distribution of 17β-HSD-immunoreactive cells (stars) and fibers (dots) in the central nervous system of *R. ridibunda*. The anatomical structures are designated on the right hemisections according to the nomenclature of Wada *et al.* (15). Ac, anterior commissure; Al, amygdala pars lateralis; Am, amygdala pars medialis; LFB, lateral forebrain bundle; MFB, medial forebrain bundle; NAS, nucleus accumbens septi; NDB, nucleus of the diagonal band of Broca; NEP, nucleus entopeduncularis; NLS, nucleus lateralis septi; NMS, nucleus medialis septi; NPO, nucleus preopticus; ON, optic nerve; PD, pallium dorsale; PLd, pallium laterale, pars dorsalis; PLv, pallium laterale, pars ventralis; PM, pallium mediale; STd, striatum, pars dorsalis; STv, striatum, pars ventralis.

(pH 7.3) and incubated overnight at 4°C with an excess of the testosterone antiserum (diluted 1:5000). Nonspecific binding was determined by substituting the testosterone antiserum with nonimmune rabbit serum (diluted 1:5000). Separation of bound and free radioactive steroids was performed by addition of 1 ml of a charcoal suspension (0.025% Norit charcoal/0.25% dextran T70 in phosphate buffer). After centrifugation, antibody-bound radioactive steroids were detected by counting 1 ml of the supernatant in a liquid scintillation counter (1217 Rackbeta LKB).

RESULTS

Immunocytochemical Localization of 17 β -HSD. The distribution of 17 β -HSD immunoreactivity in the frog brain is

schematically presented in Fig. 1. Immunopositive cell bodies were exclusively observed in the telencephalon and in the rostral part of the diencephalon. In the telencephalon, immunostaining was localized in the pallium mediale, in a group of cells bordering the inner zone of the lateral ventricles (Fig. 2*A*). Examination of these cells with a confocal laser scanning microscope revealed that the staining was homogeneously distributed in the cytoplasm and in cytoplasmic extensions (Fig. 2*B*). A few immunopositive cells were visualized in the internal zone of the pallium mediale (Fig. 2*C*). All the 17 β -HSD-immunoreactive cells exhibited thick and linear processes, characteristic of glial cells. Dense bundles of fibers were seen radiating in the pallium mediale and in the nucleus medialis septi (Fig. 2*D*). Immunoreactive processes were also found in the amygdala, the dorsal part of the striatum, the

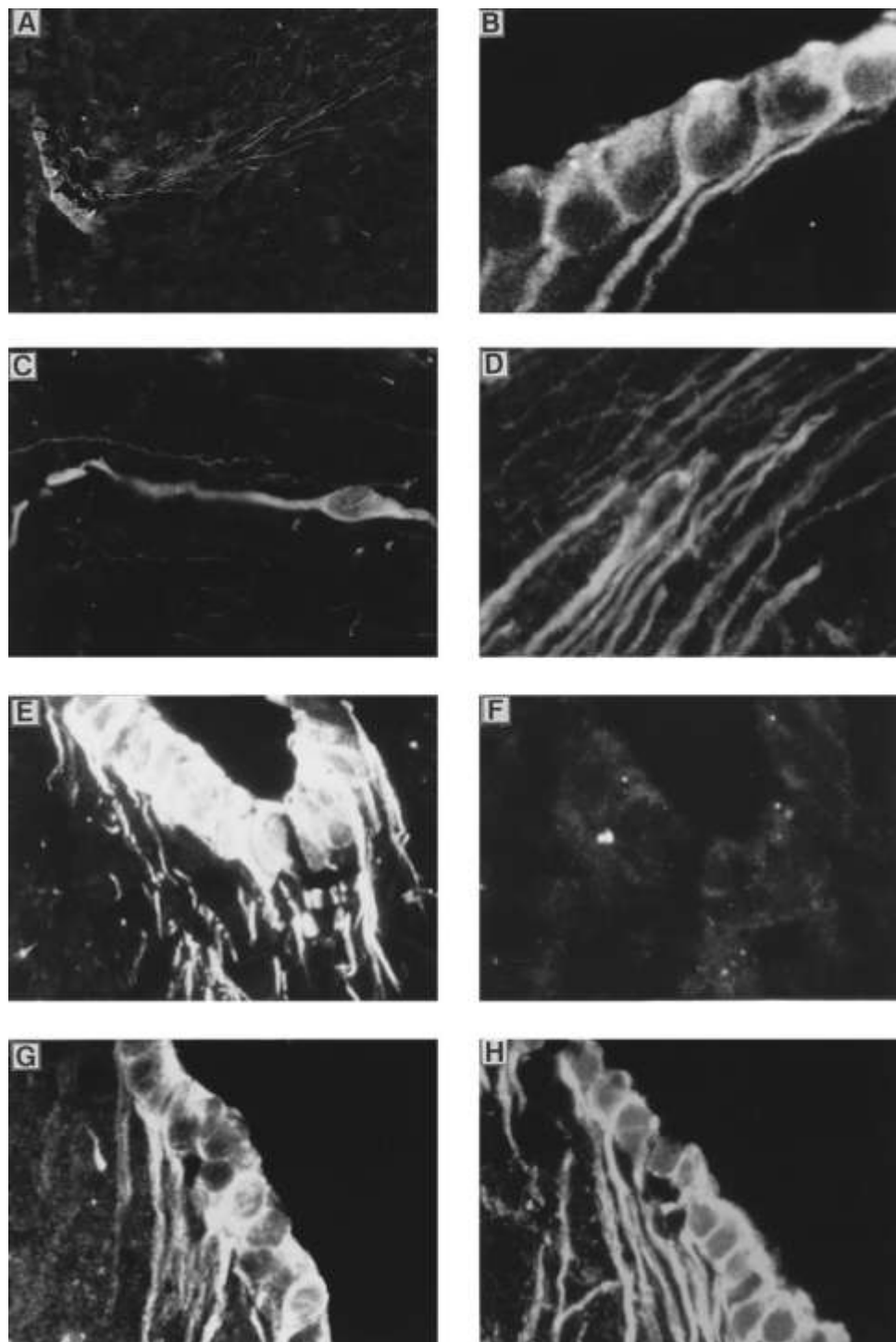


FIG. 2. 17 β -HSD-immunoreactive cells and processes in the telencephalon. (*A*) 17 β -HSD-positive gliocytes bordering the telencephalic ventricle in the pallium mediale (level +4.2 in Fig. 1). ($\times 125$.) (*B*) Confocal laser scanning microscope (CLSM) photomicrograph of 17 β -HSD-positive cells (level +3.8 in Fig. 1). ($\times 1050$.) (*C*) Isolated 17 β -HSD-positive cell in the internal layer of the pallium mediale (level +4.2 in Fig. 1). ($\times 525$.) (*D*) CLSM photomicrograph of a dense bundle of 17 β -HSD-positive processes in the nucleus medialis septi (level +3.8 in Fig. 1). ($\times 560$.) (*E* and *F*) Specificity control of the 17 β -HSD immunoreaction in the pallium mediale (level +3.8 in Fig. 1). (*E*) Positive control with nonabsorbed 17 β -HSD antiserum. (*F*) Negative control with 17 β -HSD antiserum preincubated with 17 β -HSD (10^{-6} M). (*E* and *F*, $\times 700$.) (*G* and *H*) Consecutive frontal sections through the pallium mediale (level +3.8 in Fig. 1) incubated with the 17 β -HSD antiserum (*G*) or an antiserum against the octadecanuropeptide DBI-(33-50) (*H*). (*G* and *H*, $\times 560$.)

nucleus accumbens septi, and the lateral nucleus of the septum. The medial forebrain bundle, the nucleus of the diagonal band of Broca, and the nucleus entopeduncularis contained sparse cellular processes. In the diencephalon, 17β -HSD-positive processes were only observed in the corpus geniculatus lateralis and in the lateral forebrain bundle. All other brain regions, including the mesencephalon, metencephalon, and rhombencephalon, as well as the spinal cord and pituitary, were devoid of immunoreactive elements. Preincubation of 17β -HSD antiserum with purified human 17β -HSD ($1 \mu\text{M}$) resulted in disappearance of the immunostaining (Fig. 2*E* and *F*). Labeling of consecutive sections of the telencephalon with the 17β -HSD antiserum and an antiserum raised against human DBI-(33-50) showed that 17β -HSD was localized in DBI-containing cells (Fig. 2*G* and *H*).

HPLC Analysis of Endogenous Testosterone. HPLC analysis of tissue extracts combined with radioimmunological detection revealed the presence of substantial amounts of testosterone in the frog telencephalon (512 ± 47 pg per mg of protein) and hypothalamus (115 ± 17 pg per mg of protein). The amount of testosterone in the rhombencephalon was much lower (14 ± 4 pg per mg of protein). The concentration of testosterone in the plasma was 1.4 ± 0.5 pg per mg of protein. In animals that had been castrated one week before being sacrificed, the concentration of testosterone was not significantly modified in the telencephalon (470 ± 35 pg per mg of protein) or hypothalamus (72 ± 18 pg per mg of protein). Conversely, in the rhombencephalon and blood of castrated animals, testosterone was undetectable.

GC-MS Characterization of Testosterone. Four tissue samples, each containing 300 telencephalons, and four samples of synthetic testosterone were prepurified on a Sep-Pak column and analyzed by HPLC. The peak coeluting with testosterone

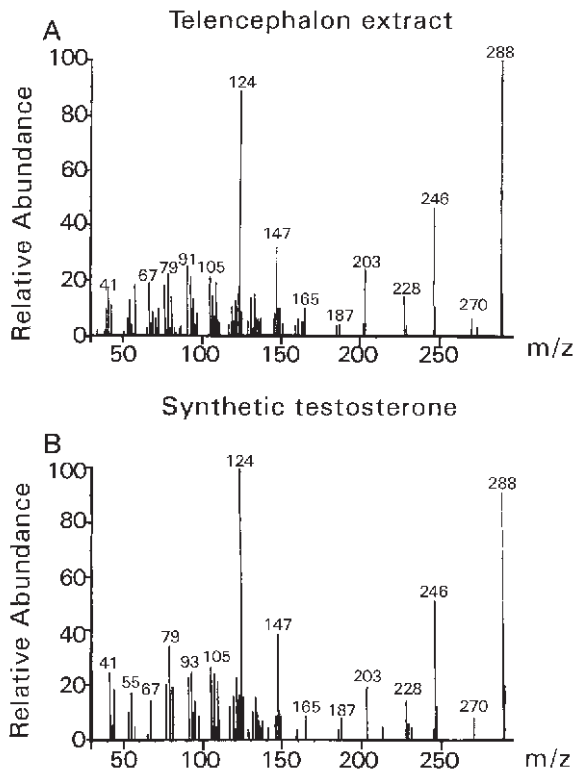


FIG. 3. GC-MS characterization of testosterone from telencephalon extracts. (A) An extract of 300 frog telencephalons was analyzed by HPLC and the peak coeluting with testosterone was identified by GC-MS; representative spectrum from four experiments. (B) Mass spectral characteristics of synthetic testosterone analyzed as in A; representative spectrum from four experiments.

was characterized by GC-MS. The endogenous steroid (Fig. 3*A*) had the same mass spectral characteristics as synthetic testosterone (Fig. 3*B*).

Conversion of [^3H] $\Delta^5\text{P}$ into Androgens. Incubation of frog telencephalon slices with [^3H] $\Delta^5\text{P}$ for 2 h yielded the formation of 15 radioactive metabolites which could be resolved by reversed-phase HPLC analysis (Fig. 4*A*). The retention times of six of the radioactive metabolites were identical to those of synthetic 5α -DHT and $17\text{-OH-}\Delta^5\text{P}$ (peak 3), DHEA (peak 5), androstenedione (peak 7), testosterone (peak 10), $17\text{-OH-}\Delta^5\text{P}$ (peak 11), and progesterone (peak 14). In control experiments, performed by replacing telencephalon slices by rhombencephalon slices, none of these metabolites was detected (Fig. 4*B*).

Immunodetection of Radiolabeled Testosterone. Telencephalon slices were incubated for 3 h with $1 \mu\text{M}$ [^3H] $\Delta^5\text{P}$, and the tissue extract was analyzed by HPLC (Fig. 5*A*). Incubation of all HPLC fractions with the testosterone antiserum made it possible to immunodetect three peaks which had the same retention times as 5α -DHT, testosterone, and $\Delta^5\text{P}$, respectively (Fig. 5*B*). Incubation of the HPLC fractions with a nonimmune rabbit serum showed that the peak coeluting with $\Delta^5\text{P}$ was attributable to the large excess of tracer which could not be totally absorbed by the charcoal suspension during the separation (Fig. 5*C*).

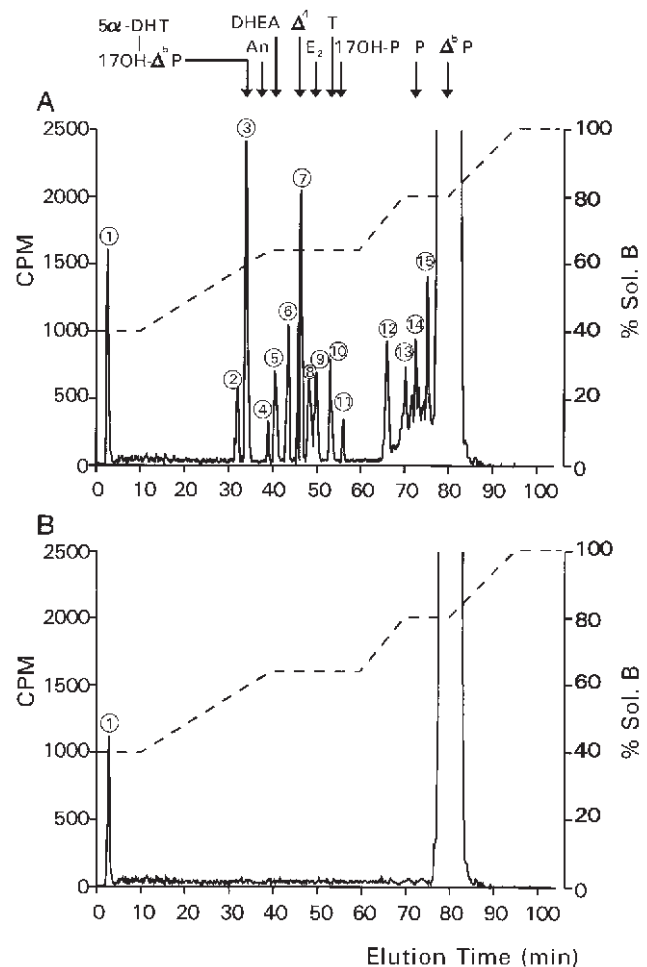


FIG. 4. HPLC analysis of radioactive steroids formed after a 2-h incubation of telencephalon slices (A) or rhombencephalon slices (B) with [^3H] $\Delta^5\text{P}$. The dashed lines represent the gradient of secondary solvent (% Sol. B). The peaks of radioactive steroids are designated by consecutive numbers (1–15). The arrows indicate the elution position of standard steroids. An, androsterone; Δ^4 , androstenedione; E_2 , estradiol; T, testosterone; P, progesterone.

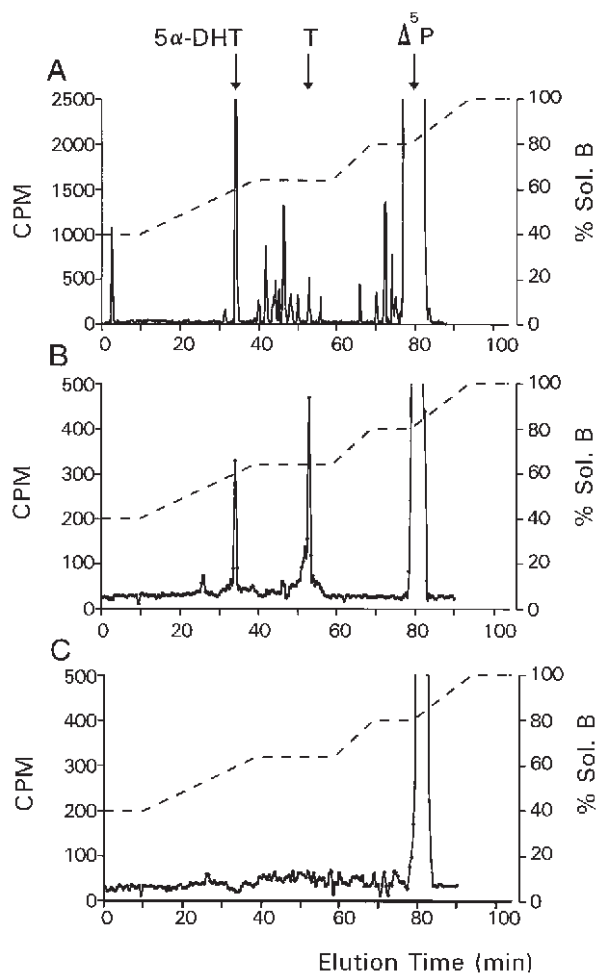


FIG. 5. Immunodetection of radiolabeled steroids in the HPLC fractions from frog telencephalon extracts. The telencephalon slices were incubated for 3 h with [^3H] $\Delta^5\text{P}$, and the radioactive metabolites were resolved by HPLC analysis. (A) Radioactivity was measured in the HPLC eluent by using a flow scintillation analyzer. (B and C) HPLC fractions were evaporated and incubated with either testosterone antiserum diluted 1:5000 (B) or nonimmune rabbit serum diluted 1:5000 (C). The radioactivity bound to the antiserum (B) or to the nonimmune rabbit serum (C) was counted. The dashed lines represent the gradient of secondary solvent (% Sol. B). The arrows indicate the elution position of testosterone (T), 5 α -DHT, and $\Delta^5\text{P}$.

DISCUSSION

The present report provides an anatomical description and cellular localization of 17 β -HSD in the central nervous system of vertebrates. The specificity of the 17 β -HSD antiserum used in this study has been controlled by immunoblot analysis of placental extracts (5). This antiserum has already been successfully applied to the immunocytochemical localization of 17 β -HSD in the human placenta (16). The preadsorption experiments and the immunoblot characterization of the antisera described herein confirmed the specificity of the immunostaining.

The 17 β -HSD-positive cells, which were organized in small groups bordering the telencephalic ventricles, exhibited the typical features of ependymal gliocytes with thick and linear processes (17). Labeling of serial sections with antisera to 17 β -HSD or DBI, a selective marker of glial cells in the amphibian brain (18, 19), confirmed that the 17 β -HSD-immunoreactive material was contained in glial elements. Endozepines such as DBI have been initially characterized as endogenous ligands of benzodiazepine receptors (20). The presence of DBI-related peptides in 17 β -HSD-containing glio-

cytes is of particular interest since endozepines have also been shown to control steroid biosynthesis in glial cells (21).

Several lines of evidence indicate that the 17 β -HSD immunoreactivity detected in glial cells is an active form of the enzyme. (i) A good correlation was observed between the distribution of 17 β -HSD-immunoreactive structures and the regional concentrations of testosterone and 5 α -DHT measured in the frog brain. Specifically, the highest concentrations of testosterone and 5 α -DHT were detected in the telencephalon where 17 β -HSD positive cells were visualized. (ii) Castration totally suppressed testosterone and 5 α -DHT in blood and in the rhombencephalon but did not affect the concentration of these two steroids in the telencephalon. Interestingly, in male mice, castration causes a marked reduction of testosterone in the central nervous system (22), suggesting that the testis is the main source of androgens in the rodent brain. (iii) Frog telencephalon slices can convert [^3H] $\Delta^5\text{P}$ into various metabolites, six of which comigrated with progesterone, 17OH-P, DHEA, androstenedione, testosterone, and 5 α -DHT. (iv) The two radioactive steroids comigrating with testosterone and 5 α -DHT were selectively immunodetected with antibodies against testosterone. (v) Finally, HPLC analysis combined with GC-MS characterization demonstrated the occurrence of authentic testosterone in frog telencephalon extracts.

The functional significance of androgen synthesis in the brain remains a matter of speculation. However, the observation that the concentrations of testosterone and 5 α -DHT are much higher in the telencephalon than in the rhombencephalon and plasma indicates that a large proportion of androgens found in the forebrain is synthesized locally and thus suggests that these endogenous steroids exert a physiological role in the brain. The presence of a high concentration of 5 α -DHT in the forebrain is of particular interest since this 5 α -reduced metabolite of testosterone is known to be the bioactive form of androgens in many tissues (23). The occurrence of 17 β -HSD-immunoreactive cells in the pallium mediale, which is part of the limbic system (24), supports the view that locally synthesized steroids may play a role in the control of behavior and sexual activity. Concurrently, the presence of 17 β -HSD-immunoreactive processes in the medial forebrain bundle and the medial part of the amygdala, in the close vicinity of gonadotropin-releasing-hormone-producing neurons (25), suggests that endogenous androgens may be involved in the neuroendocrine control of reproduction. The distribution of 17 β -HSD-immunoreactive cells in the wall of the lateral ventricles also indicates that androgens produced by ependymal cells can be released in the cerebrospinal fluid and thus may play a role in "volume transmission" (26) within the brain. Finally, the fact that glial cells express both androgen receptors (27) and a 17 β -HSD-like molecule (this study) suggests that androgens may exert paracrine and/or autocrine functions.

In conclusion, the present study has demonstrated that a discrete population of glial cells of the frog telencephalon contains 17 β -HSD-like immunoreactivity. The presence of substantial amounts of endogenous testosterone and 5 α -DHT in the telencephalon, and the ability of telencephalon slices to convert $\Delta^5\text{P}$ into DHEA, androstenedione, testosterone, and 5 α -DHT indicates that biosynthesis of androgens actually occurs in the forebrain of amphibians.

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