ORIGINAL

Effect of peripherally derived steroid hormones on the expression of steroidogenic enzymes in the rat choroid plexus

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Abstract : Peripherally derived steroids affect steroid production in the brain via the blood–brain barrier. However, steroid concentrations are lower in the cerebrospinal fluid than those in the blood, indicating restricted influx of steroids because of their metabolization by choroid plexus (CP) epithelial cells. Here, we analyzed the gene expression of steroidogenic enzymes [cholesterol side-chain cleavage enzyme (*P450scc*), 17α-hydroxylase/C17-C20 lyase (*P450c17*), 3β-hydroxysteroid dehydrogenase (*3β-HSD*), 17β-hydroxysteroid dehydrogenase type 1 (*17β-HSD1*), aromatase (*Cyp19a1*), and 5α-reductase type 1 (*5α-R1*)]. These genes were expressed to a lesser extent in the CP than in the testis and to a similar extent in the cerebral cortex. However, *P450scc* levels were higher in the CP than in the cerebral cortex, whereas *Cyp19a1* levels showed the opposite trend. We also evaluated the effects of orchiectomy and testosterone on the expression of these genes. *P450c17* and *5α-R1* levels were unaffected by orchiectomy, whereas *P450scc* and *3β-HSD* levels were increased and decreased, respectively. *Cyp19a1* expression increased upon testosterone treatment, whereas that of *17β-HSD* decreased upon orchiectomy or administration of testosterone. Immunohistochemistry analysis revealed that *17β-HSD* was expressed in the cytoplasm of CP epithelial cells. These results indicate that CP epithelial cells synthesize and convert the certain types of steroids to contribute to the homeostasis of steroids in the brain. J. Med. Invest. 68 : 238-243, August, 2021

Keywords **:** *steroidogenic enzyme, choroid plexus, orchiectomy, testosterone, immunohistochemistry*

INTRODUCTION

Steroid hormones are produced in the adrenal glands, testis, and ovaries and circulate in the blood to act throughout the body. Neurons in the central nervous system have also been reported to produce steroid hormones *de novo* (1, 2). Estrogen protects estrogen-producing neurons from oxidative stress (3, 4). Steroid hormones are lipid soluble molecules that cross membranes and affect the central nervous system. Evidence suggests that the levels of steroid hormones in the cerebrospinal fluid are lower compared to those in the blood. The blood–brain and blood–cerebrospinal fluid barriers block the entry of substances into the brain (5) and may control the inflow of steroid hormones. We predicted that the metabolism of steroid hormones in choroid plexus (CP) epithelial cells is one among the mechanisms responsible for this barrier function.

The CP plays an important role in the nutritional homeostasis of the whole brain via the cerebrospinal fluid (6). Accordingly, CP epithelial cells have been shown to constitute the blood–cerebrospinal fluid barrier, with their distal sides facing the cerebrospinal fluid and proximal ends facing the fenestrated capillary, thereby creating an interface between the cerebrospinal fluid and peripheral blood (7). The levels of D-amino acid oxidase, an enzyme involved in the metabolism of D-amino acids, are lower in the cerebrospinal fluid than those in the blood, similar to steroid hormones (8).

In this study, we conducted gene expression analysis to investigate the presence of steroid-metabolizing enzymes in the CP. Furthermore, we examined the influence of peripherally produced steroid hormones on the expression of these genes. We focused on six enzymes involved in the synthesis and metabolism of sex steroids in the CP of normal and castrated male rats : cholesterol side-chain cleavage enzyme (*P450scc*), 17α-hydroxylase/C17-C20 lyase (*P450c17*), 3β-hydroxysteroid dehydrogenase (*3β-HSD*), 17β-hydroxysteroid dehydrogenase type 1 (*17β-HSD1*), aromatase (*Cyp19a1*), and 5α-reductase type 1 (5α-R1).

EXPERIMENTAL PROCEDURES

Animals

Eight-week-old male Wistar rats were subjected to orchiectomy or sham operation. One week after the operation, orchiectomized animals were divided into 2 groups (n = 4). Rats in one group were subcutaneously administered testosterone (2 mg/kg, Sigma-Aldrich, St. Louis, MO, USA), whereas those in the other group were administered sesame oil (as a solvent) at 10:00 a.m. daily for 7 d. Sham-operated animals were treated in the same manner. All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals adopted by the Committee on Animal Research in Tokushima University (No, 11031), and were accredited by the Japanese Ministry of

Abbreviations
P450scc : cholesterol side-chain cleavage enzyme, P450c17 : 17α-hydroxylase/C17-C20 lyase, 3β-HSD : 3β-hydroxysteroid dehydrogenase, 17β-HSD1 : 17β-hydroxysteroid dehydrogenase type 1, Cyp19a1 : aromatase, 5α-R1 : 5α-reductase type 1, RT-PCR : reverse transcription-polymerase chain reaction, Tes : testis, Ad : adrenal gland, Cx : cerebral cortex, CP : choroid plexus

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Education, Culture, Sports, Science, and Technology. Every effort was taken to minimize the number and suffering of animals used throughout all experimental procedures.

Reverse transcription-polymerase chain reaction (RT-PCR)

Rats administered testosterone or sesame oil were decapitated under anesthesia [0.3 mg/kg medetomidine hydrochloride (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan), 4 mg/kg midazolam (Astellas Pharma Inc. Tokyo, Japan), and 5 mg/kg butorphanol tartrate (Meiji Seika Pharma Co., Ltd., Tokyo, Japan)] at 3 h after treatment. Subsequently, the brains, testes (Tes), and adrenal glands (Ad) were removed. The cerebral cortices (Cx) of the anterior brain and CP of the fourth ventricle were used for the experiments, whereas the olfactory bulb, hypothalamus, and thalamus were discarded (Alves *et al*., 2009). Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA). The absorption spectrum (220-350 nm) was measured with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA quantity and quality were calculated by the Beer's Law equation and determining the ratio of absorbance at 260 and 280 nm. A 260/280 ratio of 1.8-2.0 was accepted as pure for extracted RNA. We used 3 μg of total RNA for RT with the PrimeScript II 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). The synthesized cDNA was purified with a GenElute PCR Clean-UP Kit (Sigma-Aldrich). The concentration of the purified product was measured using the NanoDrop spectrometer. The PCR mixture contained 200 ng cDNA, 0.5 μM of each primer, and 1× Go Taq Green Master Mix (Promega, Madison, WI, USA) in a total volume of 25 μL. Amplification was performed using the Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The PCR cycle parameters were as follows : initial denaturation for 5 min at 95°C, denaturation for 30 s at 95°C, primer annealing for 45 s at 51-66°C, extension at 74°C for 45 s, and final extension at 74°C for 7 min. The primer sequences, annealing temperature, PCR cycles, and predicted product size are listed in Table 1. PCR products were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. Images of the generated bands were captured using a Gel Doc EZ system (Bio-Rad, Hercules, CA, USA) and analyzed using ImageJ version 1.44 software (National Institutes of Health, Bethesda, MD, USA). The mRNA levels of steroidogenic enzymes were normalized to those of glyceraldehyde 3-phosphate dehydrogenase, which was used as an internal standard.

Immunohistochemistry analysis

Orchiectomized and testosterone-administered rats were anesthetized with pentobarbital and perfused with 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany). The brains of the rats were removed and fixed overnight, after which the CP of the fourth ventricle was dissected. Frozen sections of the tissues were prepared, treated with blocking buffer (phosphate-buffered saline (FUJIFILM Wako Pure Chemical Co., Osaka, Japan) containing 3% bovine serum albumin (FUJIFILM Wako Pure Chemical Co.), 0.1% Triton X-100 (FUJIFILM Wako Pure Chemical Co.), and 0.05% sodium azide (FUJIFILM Wako Pure Chemical Co.) for 30 min, and then incubated with rabbit anti-17β-HSD1 polyclonal antibody (diluted 1 : 1000) in blocking buffer overnight at 4°C. All procedures for antigen purification (17β-HSD1), antibody production, and antibody/antigen immunoreactivity assay were performed as previously described (9). After incubation with the primary antibody, the sections were incubated with fluorescein-labeled secondary antibody for 2 h at 20°C (Alexa Fluor 488 donkey anti-rabbit IgG, diluted 1 : 1000 ; Invitrogen). The sections were rinsed in phosphate-buffered saline and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). For fluorescence microscopy, the sections were viewed under a microscope (Eclipse E800 ; Nikon, Tokyo, Japan) equipped with appropriate filter sets and a digital camera (SD-Fi1 ; Nikon). For electron microscopy, the frozen sections were incubated with 3,3′-diaminobenzidine (Dojindo, Kumamoto, Japan) after treatment with the primary antibody and ABC kit (Vector Laboratories) and fixed in 1% osmium tetroxide solution (TAAB Laboratories, Reading, UK). The sections were then stained with uranyl acetate and embedded in Epon epoxy resin (TAAB Laboratories). Ultrathin sections (80 nm) were cut and observed without lead staining.

Statistical analysis

All data have are shown as the mean ± standard deviation. Statistical analysis was performed using Student's *t*-test. $p < 0.05$ was considered to indicate statistically significant results.

Table 1. Primers used for semiquantitative reverse transcription-polymerase chain reaction (PCR)

Enzyme name	Accession No.		Primer sequence	PCR		
				Ta	cycles	Product (bp)
P450scc	NM_017286	forward	5′-AGAATTGTCCTAAACCAAGAGG-3′	60	35	579
		reverse	5′-GTGTGCCATTTCATAAAGGTTC-3′			
3β-HSD	NM_001007719	forward	5′-GCAGCACAGTTGACGTTGCAG-3′	62	32	491
		reverse	5′-AAGGCTCCAGCTGGAATCAAGG-3′			
P450c17	NM_012753	forward	5′-TTGCCACGGTGGGAGACATC-3′	66	35	357
		reverse	5′-ACTGATCTGGCTGGTCCCATT-3′			
17β-HSD	NM_054007	forward	5′-GCTGGCCAGACATGGACTCA-3′	59	33	213
		reverse	5′-AGGTTGGGGAGCATTCCAACA-3′			
Aromatase	NM_017085	forward	5′-CCATCAAGCAGCATTTGGAC-3′	53	28	368
		reverse	5′-TCCACGTCTCTCAGCGAA-3′			
5α-reductase	NM_017070	forward	5′-CGCTGTACGAGTACATTCGTC-3′	55	35	213
		reverse	5′-ACTGGCTCAAGTATCTGCTCT-3′			
GAPDH	NM_017008	forward	5′-GTGAAGGTCGGTGTGAACG-3′	55	21	300
		reverse	5′-GGTGAAGACGCCAGTAGACTC-3′			

Ta ; Annealing temperature.

RESULTS

We performed RT-PCR to evaluate the gene expression of *P450scc*, *P450c17*, *3β-HSD*, *17β-HSD1*, *Cyp19a1*, and *5α-R1* in the rat CP. The Tes expressed all tested genes, whereas the Ad expressed *P450scc*, *P450c17*, *3β-HSD*, and *5α-R1* genes but not the *17β-HSD1* and *Cyp19a1* genes (Fig. 1). *P450scc P450c17*, *3β-HSD*, *17β-HSD1*, *Cyp19a1*, and *5α-R1* were found to be expressed in the CP (Fig. 1).

We also examined the influence of peripherally derived testosterone on the expression of steroidogenic enzyme genes by semiquantitative RT-PCR. The levels of *P450scc* decreased slightly in rats subjected to orchiectomy but were not significantly different from those in the control group (Fig. 2A). The expression levels of *P450c17* and *3β-HSD* were unaffected by orchiectomy or testosterone replenishment (Fig. 2B, C). Furthermore, the gene expression of 17β-HSD1 was significantly decreased in the oil-treated group compared to in the testosterone-treated group in sham-operated mice (sham (oil) ; mean ± SD = 1.33 ± 0.19, sham (Tes) ; mean ± SD = 0.86 ± 0.08, $p = 0.037$, Fig. 2D). In contrast, the gene expression level of Cyp19a1 was significantly increased in the testosterone-treated group compared to in the oil-treated group in sham-operated mice (sham (oil) ; mean ± SD = 0.17 ± 0.24, sham (Tes) ; mean ± SD = 0.96 ± 0.32, $p = 0.028$, Fig. 2E). Finally, 5α-R1 levels remained unchanged upon orchiectomy or testosterone replenishment (Fig. 2F).

To investigate whether any of the steroidogenic enzymes is also present in the CP and to histologically confirm the results of gene expression analysis, we performed immunohistochemistry evaluation. We used an anti-17β-HSD1 antibody, which has been reported to be specific (9). Immunostaining revealed immunopositive signals in antibody-reacted tissues compared

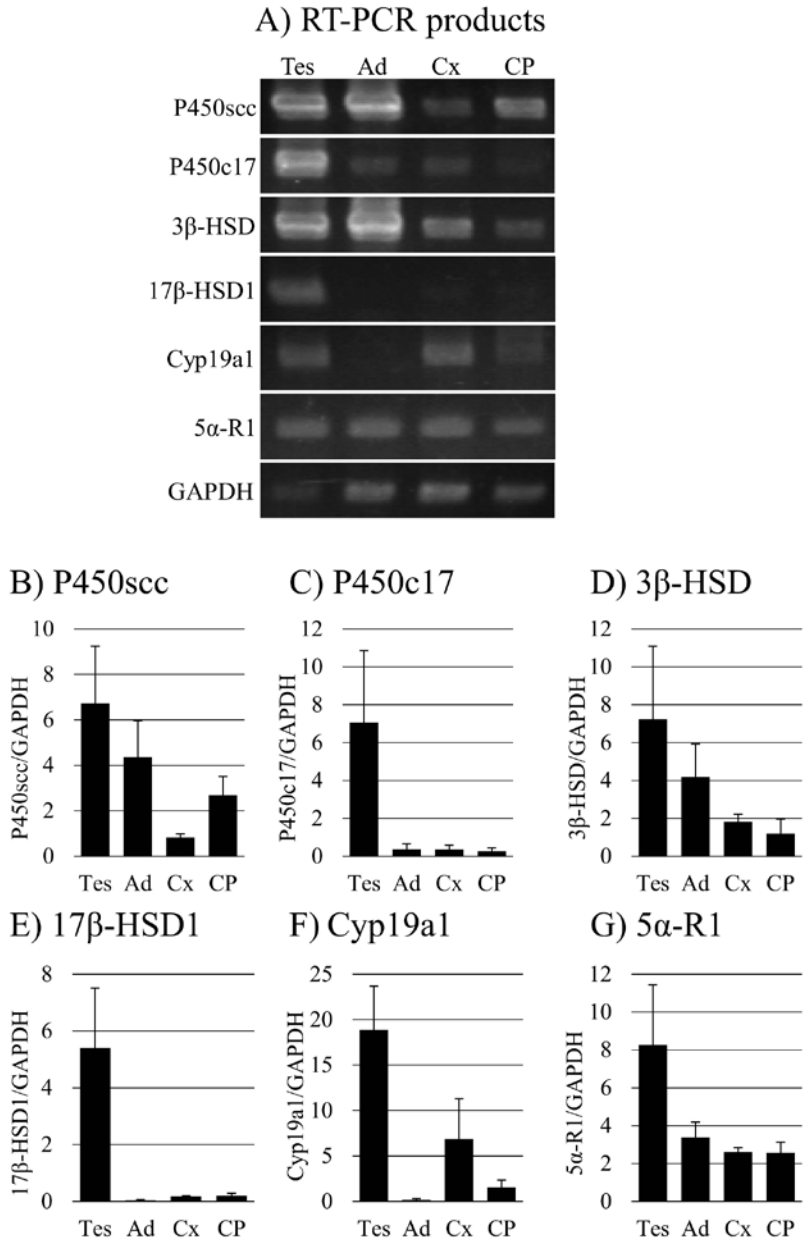

Fig. 1. The mRNA levels of steroidogenic enzymes in the rat testis (Tes), adrenal gland (Ad), cerebral cortex (Cx), and choroid plexus (CP). (A) RT-PCR products, and histograms of (B) *P450scc*, (C) *P450c17*, (D) *3β-HSD*, (E) *17β-HSD1*, (F) *Cyp19a1*, and (G) *5α-R1* comparing expression levels (all n = 4). Histograms represent the results of semiquantitative analysis of the expression of steroidogenic enzymes.

to in preimmune serum-reacted tissues. At the optical microscopy level, these signals were observed in the cytoplasm of CP epithelial cells and not in the connective tissue or blood vessels that surrounded by CP cells (Fig. 3). In addition, to observe the subcellular localization of 17β-HSD1 in the CP by ultrastructure analysis, we applied immunohistochemistry in electron microscopy. Transmission electron microscopy confirmed that 17β-HSD1 was localized to the cytoplasm (Fig. 4).

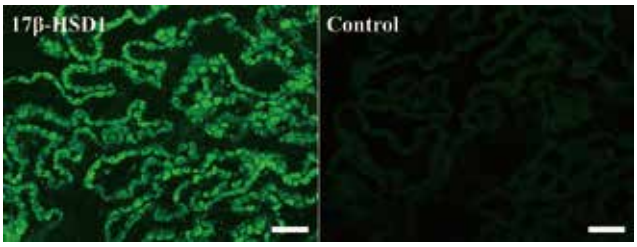

Fig. 3. Immunohistochemical analysis of rat CP in the fourth ventricle using rabbit anti-17β-HSD1 antibody and rabbit serum (preimmune). The immunoreactivity of 17β-HSD1 was detected in CP epithelial cells. Scale bar = 50 μm.

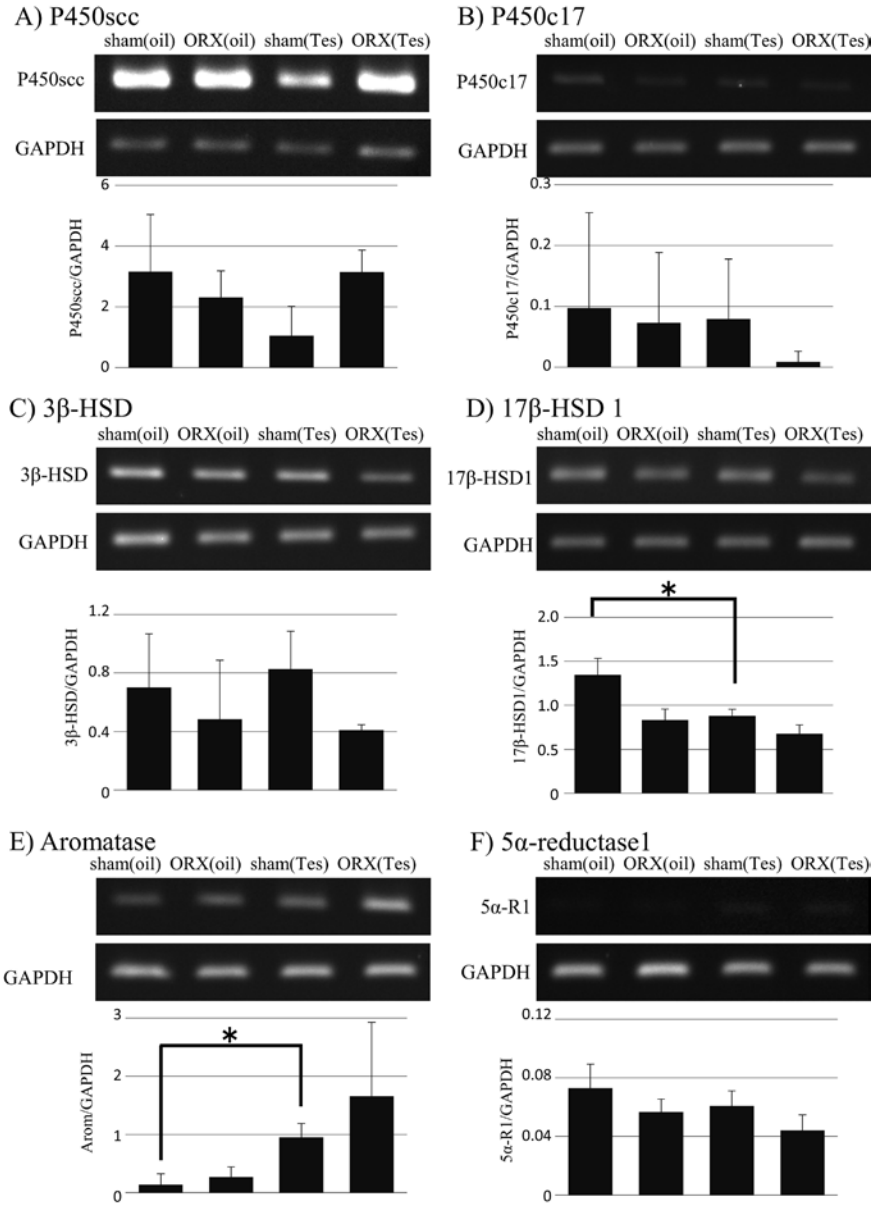

Fig. 2. mRNA levels of steroidogenic enzymes detected in the rat CP by RT-PCR with (A) *P450scc*, (B) *P450c17*, (C) *3β-HSD*, (D) *17β-HSD1*, (E) *Cyp19a1*, (F) *5α-R1*, and GAPDH-specific primers. Lane 1 : sham operation + sesame oil ; lane 2 : orchiectomy + sesame oil ; lane 3 : sham operation + testosterone ; lane 4 : orchiectomy + testosterone (all n = 4). Histograms represent the results of semiquantitative analysis of the expression of steroidogenic enzymes in CP. (A)–(C) and (F) Not significantly different from that of the control group. (D) Significantly decreased in the oil-treated group compared to the testosterone-treated group in sham-operated mice (Sham (oil) ; mean ± SD = 1.33 ± 0.19, sham (Tes) ; mean ± SD = 0.86 ± 0.08, $p = 0.037$). (E) Significantly increased in the testosterone-treated group compared to the oil-treated group in sham-operated mice (Sham (oil) ; mean ± SD = 0.17 ± 0.24, sham (Tes) ; mean ± SD = 0.96 ± 0.32, $p = 0.028$).

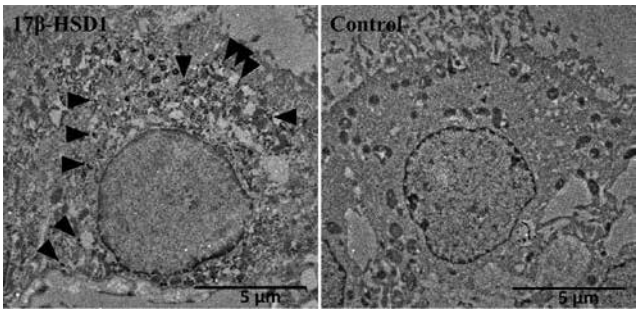

Fig. 4. Transmission electron microscopy of rat CP. Immunoelectron microscopy revealed the cytoplasmic localization of 17β-HSD1. Bar = 5 μm.

DISCUSSION

Steroid hormones selectively permeate the blood–brain barrier (10). Aldosterone and cortisol exhibit low levels of permeability, whereas progesterone, estradiol, and testosterone exhibit high potential for permeability. Hormones with high permeability are produced in the gonads and pass through the blood–brain barrier, and thus may act on neurons or glial cells. However, the correlation between steroid hormones and the CP, which is responsible for maintaining central nervous system homeostasis via the cerebrospinal fluid, remains poorly understood. We found that genes encoding enzymes involved in the metabolism of steroid hormones were expressed in the CP (Fig. 1 and 2). The levels of steroidogenic enzymes in the CP were much lower than those in the Tes (Fig. 1). Thus, we predicted that limited amounts of metabolized steroids flow from the blood to the cerebrospinal fluid. However, the expression of *P450scc* was higher than that in the Cx (Fig. 1B). Notably, P450scc, which is known to result in the production of pregnenolone from cholesterol, was present in the CP, suggesting that CP epithelial cells not only metabolize steroid hormones, but also possess the ability to *de novo* synthesize steroid hormones from cholesterol. Immunostaining (Fig. 3) and immunoelectron microscopy (Fig. 4) also revealed that 17β-HSD1 was expressed in the cytoplasm in CP epithelial cells, in accordance with previous biochemical results (11).

Taken together, we propose 3 hypotheses for the significance of steroid hormone-metabolizing enzymes in the CP. 1) Enzymes present in CP epithelial cells may act as a cerebrospinal fluid barrier, regulating the influx of steroid hormones from the blood vessels to the cerebrospinal fluid. 2) Steroid hormones may be directly synthesized by CP epithelial cells and secreted into the cerebrospinal fluid. 3) Steroid hormones discharged from the cerebrospinal fluid into the blood may be metabolized in the CP. As these hypotheses could not be verified by functional analysis, we measured the expression of steroid hormone synthesizing enzymes in primary cultured CP epithelial cells isolated from the CP of the lateral ventricle to determine whether steroid hormones were synthesized in these cells.

Androgen receptors are expressed in the brain and the expression levels were altered by steroid hormone levels (12, 13). As such, changes in the expression of steroid hormone-metabolizing enzymes in CP epithelial cells in response to administration of testosterone in this study may have contributed to the variations observed in the levels of steroid hormones in the peripheral blood. This phenomenon indicated that CP epithelial cells perceive peripheral steroids via their respective receptors and metabolize the hormones, thereby providing a potential mechanism for homeostasis of steroid hormones in the cerebrospinal fluid (Fig. 5).

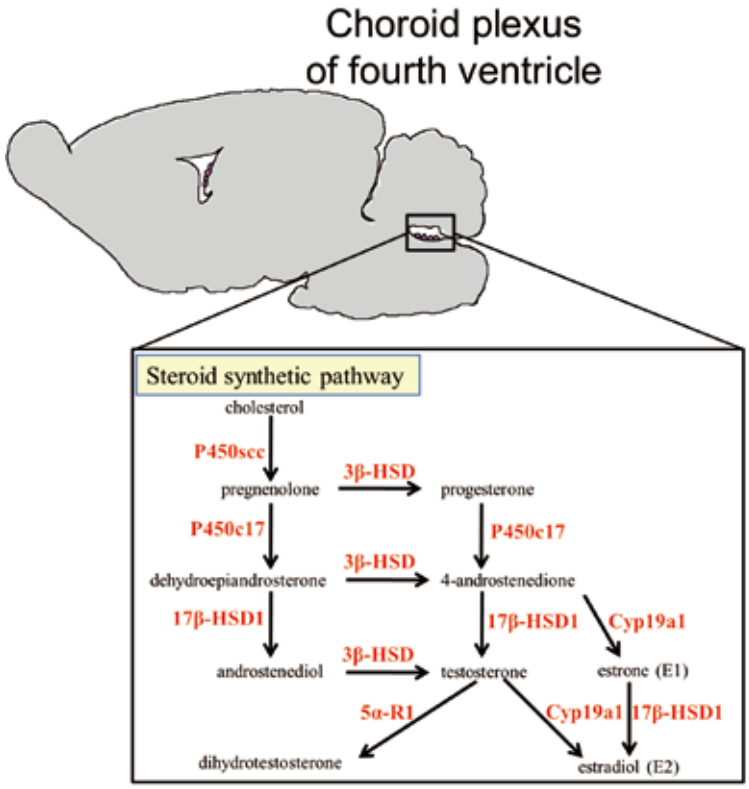

Fig. 5. Graphic abstract of steroidogenic enzymes in the rat choroid plexus. The upper half of the image shows a sagittal section of a rat. The pink structures in each ventricle represent the choroid plexus. We analyzed the expression of steroidogenic enzymes in the choroid plexus in the fourth ventricle. Enzymes involved in the steroid synthesis pathway shown in the lower half of the figure were present.

As the expression of steroidogenic enzymes in the CP was much lower than that in Tes, we considered that the metabolism of steroids in the CP was limited upon entry of the hormones into the cerebrospinal fluid from the blood. However, gene expression in the CP changed in the presence of peripheral steroid hormones. Although the gene expression profiles in the CP and Cx were similar for most genes, the expression of *P450scc* and *Cyp19a1* in the CP differed from that in the Cx. Thus, among the steroids required in the brain, some are synthesized as precursors in the CP and may eventually be converted in the brain.

DECLARATION OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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