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Cytochrome P450 Side-Chain Cleavage Enzyme in the Cerebellar Purkinje Neuron and Its Neonatal Change in Rats*

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ABSTRACT

Neurosteroids are *de novo* synthesized in the nervous system through mechanisms at least partly independent of peripheral steroidogenic glands. However, the concept of neurosteroidogenesis in neurons is not clear in mammalian brains. The present study identified the presence of cytochrome P450scc in the rat Purkinje cell, a typical cerebellar neuron. Immunohistochemical analysis with the antibody against the purified bovine adrenal P450scc showed an immunoreaction restricted to somata and dendrites of the Purkinje cells in adult cerebella. Preadsorbing the antibody with P450scc resulted in a complete absence of the immunoreaction. The antibody against inositol triphosphate receptor, a marker of the Purkinje cell, recognized P450scc-immunoreactive cerebellar cells that showed no immunoreaction with glial fibrillary acidic protein, a specific marker of glial cells. Expression of the P450scc-like protein in the cerebellum was verified by Western blot analysis, and cerebellar P450scc messenger RNA, by RT-PCR analysis in adulthood. On the other hand, P450scc-immunoreactive cells were found to scatter throughout the cerebellum at 0 day of age, before the differentiation of the first Purkinje cells, while the site of expression of this protein was localized only in somata of Purkinje cells at 3 days of age. Immunoreactive dendrites of the Purkinje cell spread into the molecular layer during neonatal development concurrently with its maturation. The intensity of the immunoreaction did not change during neonatal life. Expression of the cerebellar P450scc messenger RNA was also detected after birth, and the level was almost constant during neonatal life. A specific RIA indicated that the pregnenolone concentration was unexpectedly high at 0 day and decreased until 7 days. The total amount of pregnenolone in the cerebellum was almost constant from 0–7 days and increased during 7–21 days concurrently with the cerebellar development. In contrast, the pregnenolone sulfate ester level was low and did not significantly change among the developmental stages.

These results suggest that steroidogenic enzyme P450scc appears in the rat Purkinje cell immediately after its differentiation. The expression of this enzyme may remain during neonatal development and in adulthood. (*Endocrinology* **139:** 137–147, 1998)

STEROID hormones supplied by peripheral steroidogenic glands regulate neuronal functions during development and in adulthood. Peripheral steroids cross the blood-brain barrier as a result of their lipid solubility and act on brain tissues through intracellular receptor-mediated mechanisms that regulate the transcription of specific genes (1, 2). Therefore, the brain is considered to be a target site of peripheral steroids.

However, new findings have been obtained that pregnenolone and dehydroepiandrosterone, as unconjugated steroids, and their fatty acid or sulfate esters accumulate within the brain in several mammalian species (3–10) and an avian species (11–13). The brain content of these steroids is almost constant even after the removal of peripheral steroids, *e.g.* adrenalectomy, castration, and hypophysectomy, suggesting that the brain can synthesize steroids *de novo* (3–6, 8, 9, 11, 13). Such steroids synthesized in the brain are called neurosteroids (14). Indeed, it has been demonstrated that certain structures in the mammalian and avian brain have the capacity to metabolize cholesterol to pregnenolone (11, 13, 15–21). The cytochrome P450scc side-chain cleavage enzyme (P450scc) cleaves cholesterol to form pregnenolone (for a review, see Ref. 22). Recent studies further indicated that both P450scc protein and its messenger RNA (mRNA) are expressed in the rat brain (14, 16, 17, 19, 20, 23, 24). Neurosteroids are thought to mediate their actions through ion-gated channel receptors, such as γ-aminobutyric acid A and *N*-methyl-D-aspartate (25–33), rather than through classic nuclear steroid receptors. Dehydroepiandrosterone inhibits aggressive behavior of castrated male mice against lactating female intruders (5, 6, 8, 34), but its activity is probably not related to the conversion into testosterone and estradiol (34).

In mammals, glial cells are considered to play a major role in neurosteroid formation and metabolism in the brain. P450scc has been found in the white matter throughout the rat brain (14). It has further been shown that both oligodendrocytes and astrocytes are the primary site for pregnenolone synthesis (15–20). However, the concept of neurosteroidogenesis in neurons is still unclear in the mammalian brain, although neuronal P450scc expression has been reported in the rat nervous system, such as neurons in the retinal ganglion, sensory neurons in the dorsal root ganglia, and motor neurons in the spinal cord (24, 35). On the other hand, we have recently demonstrated that the avian brain also possesses cytochrome P450scc and produces pregnenolone and its sulfate ester, by biochemical and immunochemical approaches (11). In addition, our immunohistochemical studies with avian brain have shown that an intense immunoreac-

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tion with the polyclonal antibody directed against the purified bovine adrenal P450scc is present in soma and dendrites of the Purkinje cell, a typical cerebellar neuron (12, 13).

With these findings as a background, we first investigated the presence of P450scc in the cerebellar Purkinje cell using the mammalian species, *i.e.* rats. The second purpose of this study was to determine neonatal changes in P450scc located in the Purkinje cell. Finally, diurnal changes in the cerebellar P450scc expression were examined as a possible physiological change.

Materials and Methods

Animals

Male rats of the Fisher strain maintained in this laboratory were used. They were housed in a temperature-controlled room (25 ± 2 C) under daily photoperiods of 14-h light, 10-h dark cycles (lights on at 0006 h) and were given food and tap water *ad libitum*. Males at the ages of 0, 3, 7, 14, and 21 days and sexually mature males at the age of 2 months were prepared as subjects in this study. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Hiroshima University, Japan.

Immunohistochemical analysis with P450scc antibody

In the present immunohistochemical experiment, 30 male rats at various ages (n = 5 at each age) were deeply anesthetized with a chloroform and then perfused transcardially with PBS [0.1 M phosphate buffer (PB); 0.14 M NaCl, pH 7.3] followed by fixative solution (4% paraformaldehyde in 0.1 M PB). After dissection from the skull, brains were postfixed for 24–48 h in the same fixative solution at 4 C and then soaked in a refrigerated sucrose solution (30% sucrose in 0.1 M PB) until they sank. All cerebella were frozen-sectioned sagittally at 40 μm thickness on a cryostat at −18 C. Every third section was grouped in a single batch of ice-cold PBS; thus we obtained three independent series of adjacent sections. Only one of these series of sections was used for immunohistochemical staining with cytochrome P450scc, while the remaining two series were used for control staining of immunohistochemistry and for Nissl-staining, respectively.

The sections were processed according to the avidin-biotin-peroxidase complex (ABC) immunocytochemical technique with the floating method described previously (12, 36, 37). 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 primary antiserum directed against the bovine adrenal cytochrome P450scc at a dilution of 1:1,000 for 36–48 h at 4 C. The anti-P450scc serum was raised in a rabbit (38) using purified cytochrome P450scc from bovine adrenocortical mitochondria (39). The details of the characterization of this serum are given elsewhere (11, 12, 38). To block nonspecific binding components, the anti-P450scc serum was also preincubated with PBS containing 0.5% bovine liver acetone powder (Sigma, St. Louis, MO) and 1% BSA for 12–18 h at 4 C as described previously (12, 36, 37). Several concentrations of the antiserum from 1:1,000 to 1:4,000 were examined, and a solution of 1:1,000 proved most satisfactory (12). The primary immunoreaction was followed by a 60 min-incubation with biotinylated antirabbit IgG (10 μg/ml) (Vector Laboratories, Burlingame, CA) and finally by a 60 min-incubation with avidin-biotin complex (Vectastain ABC Elite kit, Vector Laboratories). Immunoreactive products were detected by immersing the sections for 2–7 min in a diaminobenzidine (DAB) solution (0.05% DAB in PBS containing 0.3% H_2O_2).

The specificity of the anti-P450scc serum was assessed by a substitution of the control serum for the primary antiserum; in this control serum, the antibody (1:1,000 dilution) was preadsorbed by incubation with the purified antigen in a saturating concentration (10 μg P450scc/ml) for 12–18 h before use. The sections were incubated with this control serum, employing the same procedure for the anti-P450scc serum. The localization of immunoreactive cell bodies and fibers in the rat cerebellum was studied using an Olympus BH-2 microscope.

Identification of cell type of immunoreactive cells

To identify the cell type showing P450scc-like immunoreactivity, immunohistochemical analyses with three kinds of antibodies were subsequently performed using five adult males. One of these antibodies was against P450scc, while the remaining two antibodies were prepared as reference stainings for deciding the cell type: 1) one was against inositol triphosphate (IP_3) receptors that present abundantly in Purkinje cells, and 2) the other was against glial fibrillary acidic protein (GFAP) as a specific marker protein of glial cells. As the IP_3 receptor antibody, a purified IgG fraction of the monoclonal mouse antibody that cross-reacts with IP_3 receptors (Accurate Chemical & Scientific Co., Ltd., Westbury, NY) was used in this study. A purified IgG fraction of the polyclonal rabbit antibody directed against the purified bovine GFAP (Dako Co., Ltd., Glostrup, Denmark) was used as the GFAP antibody. It has been previously confirmed that these two reference antibodies cross-react with each rat antigen.

Fixation and immunohistochemistry were carried out in the same manner mentioned above. In brief, adjacent serial sections (40 μm thickness) were incubated with the anti-P450scc (1:1,000 dilution), the anti-IP_3 receptor (1:50 dilution), and the anti-GFAP (1:100 dilution), respectively. After the incubation, immunoreactive products were detected with the avidin-biotin kit (Vectastain Elite kit, Vector Laboratories) followed by DAB reaction.

Western immunoblot analysis with P450scc antibody

To detect cytochrome P450scc in the rat cerebellum, Western immunoblot analysis with the antibody against bovine P450scc was conducted after SDS-PAGE of tissue homogenates. Four adult males were killed between 1000 and 1200 h. In each animal, several brain regions including the cerebellum were immediately excised and placed on ice. The testis was used as a control tissue for Western immunoblot analysis because it was regarded as a classical steroidogenic organ. The tissues in each animal were separately homogenized in 4 vol of ice-cold sample buffer containing 0.05% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 1 mM phenylmethylsulfonylfluoride and centrifuged at 15,000 × *g* for 20 min. The supernatant was concentrated by precipitation with 30–50% saturation of ammonium sulfate. Proteins derived from each tissue were subjected to 10% SDS-PAGE, and then Western immunoblotting was performed according to our previous methods (11–13). In brief, after transfer onto polyvinylidene fluoride membranes (Immobilon-P, Millipore Co., Bedford, MA), the blot was probed with the anti-P450scc antibody and followed by incubation with biotinylated goat antirabbit IgG (Vector Laboratories). Finally, the membrane was incubated with streptavidin-horseradish peroxidase complex (Amersham International plc, Little Chalfont, Buckinghamshire, UK). The protein bands were detected by ECL Western blotting detection reagents (Amersham International plc). Proteins were measured by the BCA protein assay kit (Pierce, Rockford, IL) with BSA as a standard.

RT-PCR analysis of P450scc mRNA

To determine expression of the mRNA encoding for rat P450scc in the cerebellum, RT-PCR analyses were performed using rats in adulthood and during neonatal development. In this experiment, 24 male rats at various ages (n = 4 at each age) were also killed between 1000 and 1200 h. Total RNA of each cerebellum (all observed ages) as well as other brain regions (only 2 months) was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method (40). Total RNA contains ribosomal RNA and mRNA. In our experiments, the average amount of the total RNA extracted from one cerebellum was 69 μg at 0 day, 97 μg at 3 days, 137 μg at 7 days, 336 μg at 14 days, 349 μg at 21 days, and 244 μg at 60 days. Thirty micrograms of total RNA were reverse transcribed using Oligo dT primer and RT in a 60-μl reaction volume for 1.5 h at 37 C. The reaction mixture was composed with 30 μg of total RNA, 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM deoxynucleoside triphosphate (dNTP) mix, 1.5 μg of Oligo $dT_{12–18}$ (Pharmacia, Uppsala, Sweden), 15 U of ribonuclease inhibitor (Wako, Osaka, Japan), and 400 U of moloney murine leukemia virus transcriptase (GIBCO BRL, Burlington, Canada). After the reaction was stopped by incubating at 67 C for 10 min, the cDNA was ethanol precipitated and redissolved in 30 μl of distilled water. For PCR, an aliquot

of the cDNA solution corresponding to 0.5 μg of initial total RNA was used as template in a 25-μl reaction mixture. The PCR mixture contained cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mix, 12.5 pmol of each primer, and 1 U of rTaq DNA polymerase (TOYOBO, Osaka, Japan). After denaturation at 95 C for 3 min, the mixture was subjected to 30 thermal cycling in a programmed temperature control system (PC700; ASTEC, Fukuoka, Japan) as follows: denaturation at 93 C for 1 min, primer annealing at 60 C for 1 min, and extension at 72 C for 1 min. After the thermal cycling, the mixture was additionally incubated at 72 C for 10 min. A 10-μl aliquot of each sample was electrophoresed through a 1.5% agarose gel.

To confirm the identity of the amplified fragment, the gels were applied to Southern analysis with a digoxigenin-labeled oligonucleotide probe, corresponding to the internal sequence of the target gene. Digoxigenin DNA labeling and detection were performed according to the recommendations of the manufacturer (Boehringer, Vienna, Austria). Oligonucleotides used as PCR primer and probe for mRNA detection, which were based on nucleotide sequences of rat P450scc (41) and rat β-actin (42), were as follows: P450scc sense primer 5′-TCAAAGCCAG-CATCAAGGAG-3′ (nucleotide number 1141–1160 in Ref. 41), P450scc antisense primer 5′-GCAGCCTGCAATTCATACAG-3′ (nucleotide number 1594–1613 in Ref. 41), P450scc probe 5′-TTCTCAGGCATCAG-GATGAG-3′ (nucleotide number 1506–1525 in Ref. 41), β-actin sense primer 5′-GAGACCTTCAACACCCCAGC-3′ (nucleotide number 2167–2186 in Ref. 42), and β-actin antisense primer 5′-CACAGAGTACT-TGCGCTCAG-3′ (nucleotide number 3004–3023 in Ref. 42). The P450scc sense and antisense primers give 473 bp amplified fragment located in exon 6 to exon 9 of P450scc gene. The β-actin primers give 645 bp amplified fragment located in exon 3 to 6. RT-PCR analyses were repeated at least four times using independently extracted RNA samples from different animals.

RIAs of pregnenolone and its sulfate ester

To measure levels of pregnenolone and its sulfate ester in the cerebellum during neonatal development and in adulthood, 64 male rats at various ages were killed (n = 16 at 0 and 3 days, n = 12 at 7 days, n = 8 at 14 and 21 days, n = 4 at 2 months). The time lapse between the beginning and the end of the killing did not exceed 2 h, and this was always performed between 1000 and 1200 h. Trunk blood was collected into heparinized tubes and centrifuged at 1,800 × *g* for 20 min at 4 C. Plasma was stored at −80 C until assayed for pregnenolone and its sulfate ester. To secure sufficient volume of plasma for assay in younger rats, plasma from one to four animals was pooled as a sample. The assays of pregnenolone and pregnenolone sulfate ester were performed on four pooled samples at each age. Immediately after the blood collection, cerebella were taken out and weighed. Then, cerebella from one to four rats were also pooled as a sample, frozen in liquid nitrogen, and stored at −80 C. The number of cerebellar samples was also four at each age.

Extraction of unconjugated steroids or steroid sulfates was performed according to the previous method (4, 11, 13). Cerebella were homogenized in 5 ml ice-cold PBS (pH 7.6) with a Teflon-glass homogenizer. Plasma (100–200 μl) was diluted with 5 ml cold PBS. Cerebellar and plasma samples were applied to steroid extraction. To estimate the recovery of the unconjugated steroid during the extraction, 1,500 cpm of [7-^{3}H] pregnenolone was added to the samples with 5 ml ethyl acetate. The tubes were stirred for 30 min and centrifuged at 3,000 × *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 put to dryness as the assay samples for pregnenolone. On the other hand, the pH of the water phase was decreased to 1 with 30 μl sulfuric acid, and saturated sodium chloride was added as a final concentration of 20%. To calculate the recovery of the steroid sulfate ester, 1,500 cpm of [7-^{3}H]dehydroepiandrosterone sulfate ester was then added to the mixture. Extraction with ethyl acetate in the water phase was again performed as described above. Steroid sulfates were contained in this extract and solvolyzed in 10 ml 95% ethyl ether at 37 C overnight. The hydrolyzed steroids were washed once with 3 ml of 1 N NaOH and twice with 3 ml of water and put to dryness as the assay samples for pregnenolone sulfate ester. The dried residues were dissolved in 1 ml PBS containing 0.1% gelatin. Each aqueous solution obtained from both extracts of organic and water phases was divided into two aliquots: one aliquot for the recovery measurement, the other for the measurement of pregnenolone or its sulfate ester.

To measure the concentrations of pregnenolone and its sulfate ester, aliquots from both extracts of organic and water phases were applied to the pregnenolone RIA (4, 11, 13, 43, 44) using the antiserum to pregnenolone (Radioassay Systems laboratories, Inc., Immuchem Corp., Carson, CA) and [7-^{3}H]pregnenolone (specific activity, 23.5 Ci/mmol, New England Nuclear, Boston, MA). The pregnenolone assay was performed without chromatographic purification of pregnenolone, and the first antiserum used in the present experiment cross-reacted with pregnenolone sulfate at 50%, 17α-hydroxypregnenolone at 2%, and dehydroepiandrosterone less than 0.01%. Separation of bound and free steroids was performed by centrifugation after reaction with the IgG SORB (The Enzyme Center Inc., Malden, MA). The least detectable amount was 0.1 ng/ml, and intraassay variation was less than 7%. The precision index (λ) of a linear portion of the competition curve, which was computed according to the method described previously (11, 45), was 0.037 in the assay.

Statistical analysis

Results of the RIA were expressed as the mean ± SEM. Comparisons of changes in steroid concentrations and total steroid amounts in the cerebellum between different developmental stages were made by Student's *t* test.

Results

Localization of P450scc-like immunoreactive cells in the adult cerebellum

In the adult male rat, cytochrome P450scc-like immunoreactivity was present in the cerebellar cortex (Fig. 1). As shown in Fig. 1, a and b, an intense immunoreaction with the antibody against bovine adrenal P450scc was restricted to large cell bodies lying at a narrow zone between the molecular and granular layers and to fibers spreading into the molecular layer. The distribution of immunoreactive cell bodies and fibers in the cerebellar cortex was coincident with the location of somata and dendrites of Purkinje cells, characterized by the immunohistochemical and Nissl-stainings (Fig. 1, a and c). Furthermore, in their somata and dendrites, a most intense immunoreaction 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. 1b).

Preadsorbing the antibody with an excess of purified P450scc antigen (10 μg/ml) resulted in a complete absence of P450scc-like immunoreactivity in all of the positively stained cells in the cerebellum (Fig. 1, a and d). Therefore, it is probable that the observed immunoreaction is due to the antibody recognizing endogenous cytochrome P450scc-like protein.

Identification of the cell type of P450scc-like immunoreactive cells in the adult cerebellum

As shown in Fig. 1, P450scc-like immunoreactivity in the cerebellum was suggested to be located in the somata and dendrites of Purkinje cells. To confirm this finding, we further performed the immunolabelings using three kinds of antibodies against P450scc, IP_3 receptor, and GFAP. The antibody against IP_3 receptor, which is considered to be a marker protein of the Purkinje cell, recognized P450scc-like immunoreactive cells (Fig. 2, a and b). In contrast, the antibody against GFAP, a specific marker protein of glial cells,

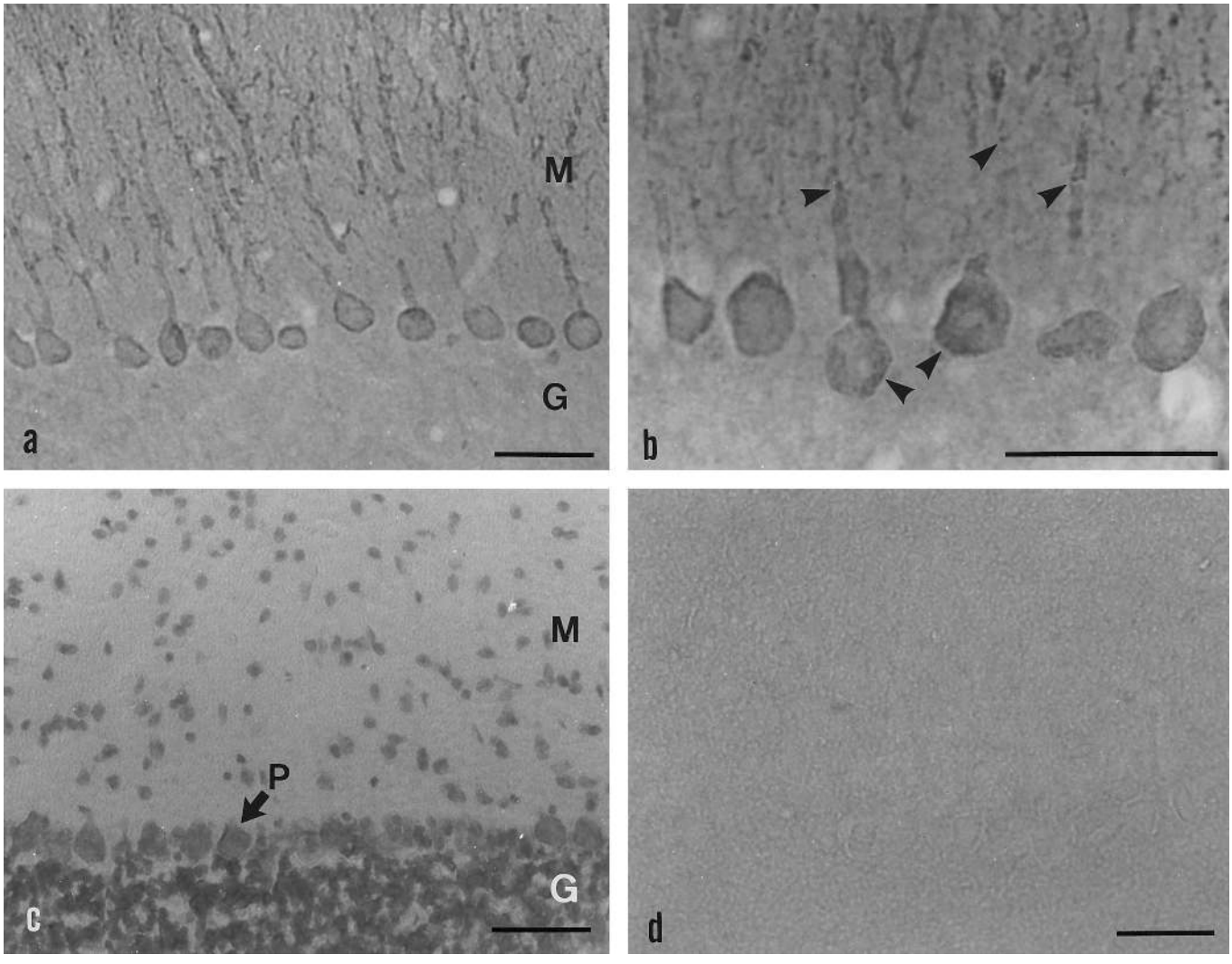

FIG. 1. Immunohistochemical staining with the antiserum to cytochrome P450scc (a and b) in the molecular (M) layer and a narrow cell layer between the molecular and granular (G) layers in the cerebellar cortex of the adult male rat. The P450scc antiserum preincubated with a saturating concentration of purified antigen (d) was substituted for the primary antibody, as a control. Histology of the cerebellar cortex was shown by Nissl staining (c). Panels a, c, and d are of the same low magnification, and panel b is of high magnification. The *arrow* indicates the Purkinje cell (P) (c). Immunoreactive cell bodies and fibers were present in the area coincident with the location of Purkinje cells (a and c). The *arrowheads* show intensive reaction granules within the immunoreactive cell bodies and fibers (b). *Bars* = 50 μm. Immunohistochemical experiments were repeated independently five times using different animals and indicated the same results.

stained a substantial number of small cells in the granular and molecular layers, but did not stain P450scc-like immunoreactive cells (Fig. 2, a and c). These results taken together suggest that P450scc-like immunoreactive cells are not glial cells and are identified as Purkinje cells, a typical cerebellar neuron.

Western immunoblot analysis of P450scc-like protein in the adult cerebellum

To determine the presence of P450scc-like protein in the cerebellum, Western immunoblot analysis with the antibody raised against bovine adrenal P450scc was performed using the cerebellum and other brain regions of the adult male rat. As illustrated in Fig. 3, a protein band derived from the cerebellum as well as other brain regions was recognized by the antibody. The intensity of immunoreaction in all brain regions was lower than in the testis (Fig. 3). Electrophoretic mobility of a protein band in the brain and testis was almost the same as that of the purified bovine adrenal P450scc (Fig. 3). The molecular mass of this immunoreactive protein was approximately 54 kDa judging from its electrophoretic mobility. This value was the same as the molecular mass of rat P450scc in the previous study (41).

RT-PCR analysis of P450scc mRNA in the adult cerebellum

We further examined P450scc mRNA expression in the cerebellum. Total RNA was extracted from several brain regions and the testis of the adult male rat and subjected to RT-PCR. The initial RNA amount provided to RT-PCR was adjusted spectrophotometrically. RT-PCR for β-actin was performed as a control experiment, and cDNA amount used in the control RT-PCR was reduced to 0.2 ng to avoid sat-

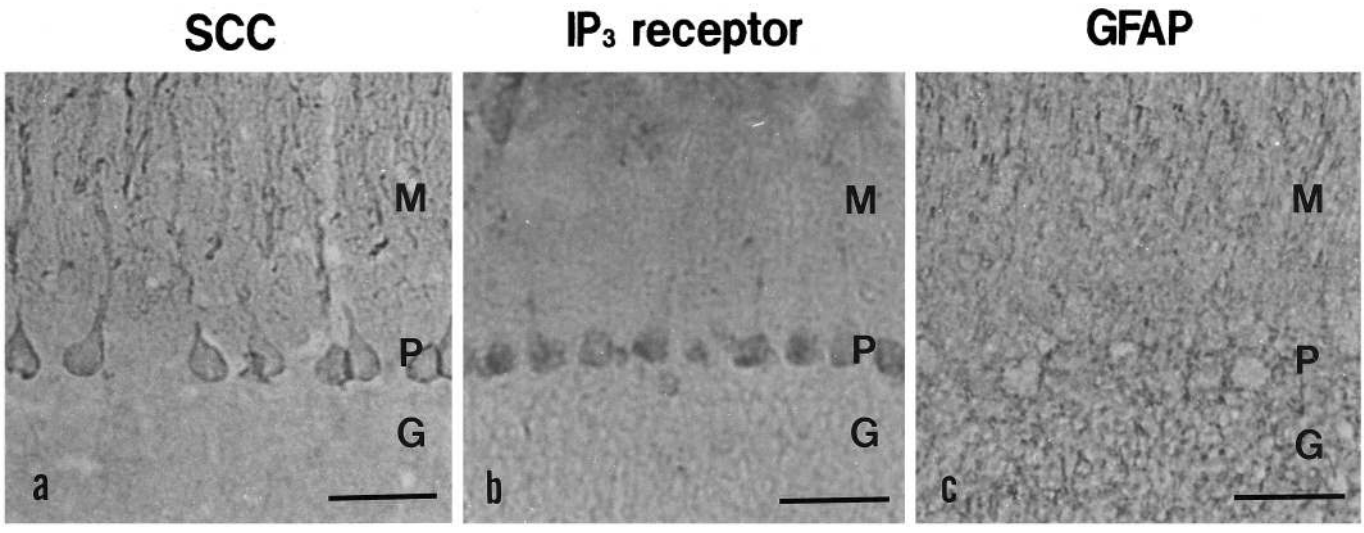

FIG. 2. Immunohistochemical staining with the antiserum to cytochrome P450scc (a), IP_3 receptor (b), or GFAP (c) in the cerebellar cortex of the adult male rat. The antiserum to IP_3 receptor (b) or to GFAP (c) was used as a specific marker of Purkinje cells or glial cells. P, Purkinje cell layer; M, molecular layer; G, granular layer in the cerebellar cortex. *Bars* = 50 μm.

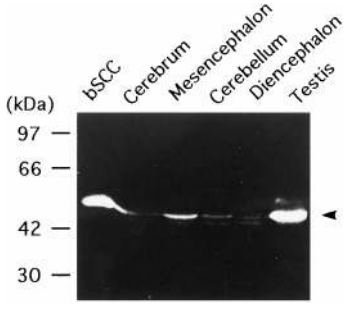

FIG. 3. Western immunoblot analysis of P450scc-like protein in the cerebellum as well as other brain regions in the adult male rat. The testis was also used as the positive control. Proteins of these tissues were treated with 2.5% SDS and electrophoresed on 10% polyacrylamide gels. Each lane contained 100 μg proteins of the respective tissues. Purified bovine adrenal P450scc (bSCC) of 1.7 fmol served as a reference marker. Western blotting was performed as described in *Materials and Methods*. The *arrowhead* indicates P450scc-like protein band. Western immuoblot experiments were repeated four times using independently extracted protein samples from different animals and indicated the same results.

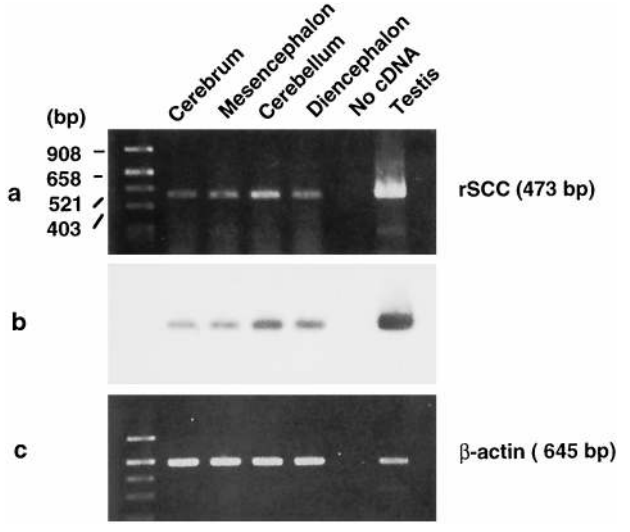

FIG. 4. RT-PCR analysis of P450scc mRNA in the cerebellum as well as other brain regions in the adult male rat. *Upper panel* (a) shows a result of the gel electrophoresis of RT-PCR products for rat P450scc (rSCC), and *middle panel* (b) shows an identification of the band by Southern hybridization using digoxigenin-labeled oligonucleotide probe for rSCC. cDNA corresponding to 0.5 μg total RNA extracted from each tissue was used for a PCR reaction, and the 2/5 was applied on one lane. The lane labeled "No cDNA" was performed without template as the negative control. *Lower panel* (c) shows a result of the RT-PCR for β-actin as the internal control, in which cDNA corresponding to 0.2 ng total RNA was used as template to avoid saturation of amplification. RT-PCR experiments were repeated four times using independently extracted RNA samples from different animals and indicated the same results.

uration of amplification (Fig. 4c). As shown in a result of gel electrophoresis of the RT-PCR product for P450scc gene (Fig. 4a), a single band corresponding to P450scc mRNA size, but not P450scc genomic DNA size, was detected in the cerebellum as well as other brain regions. Serial Southern hybridization confirmed that this band was P450scc mRNA specific (Fig. 4b). The density of the band in each brain region was lower than that in the testis (Fig. 4).

Neonatal change in P450scc-like protein in the cerebellar Purkinje cell

The following questions were asked in the next series of experiments. First, when during neonatal development does P450scc-like protein appear in the Purkinje cell? Second, what is the change in P450scc-like protein in the developing Purkinje cell? To obtain the answer to the first question, immunohistochemical analysis with the anti-P450scc serum was performed using male rats at the ages of 0, 3, and 7 days. It has been previously confirmed that in the rat, Purkinje cells differentiate in the cerebellum at 3 days of age (46, 47). Abundant P450scc-like immunoreactive small cells scattered throughout the cerebellum at 0 day of age (Fig. 5a). Such an immunoreaction at 0 day of age was completely abolished when the immune serum was immunoadsorbed by the

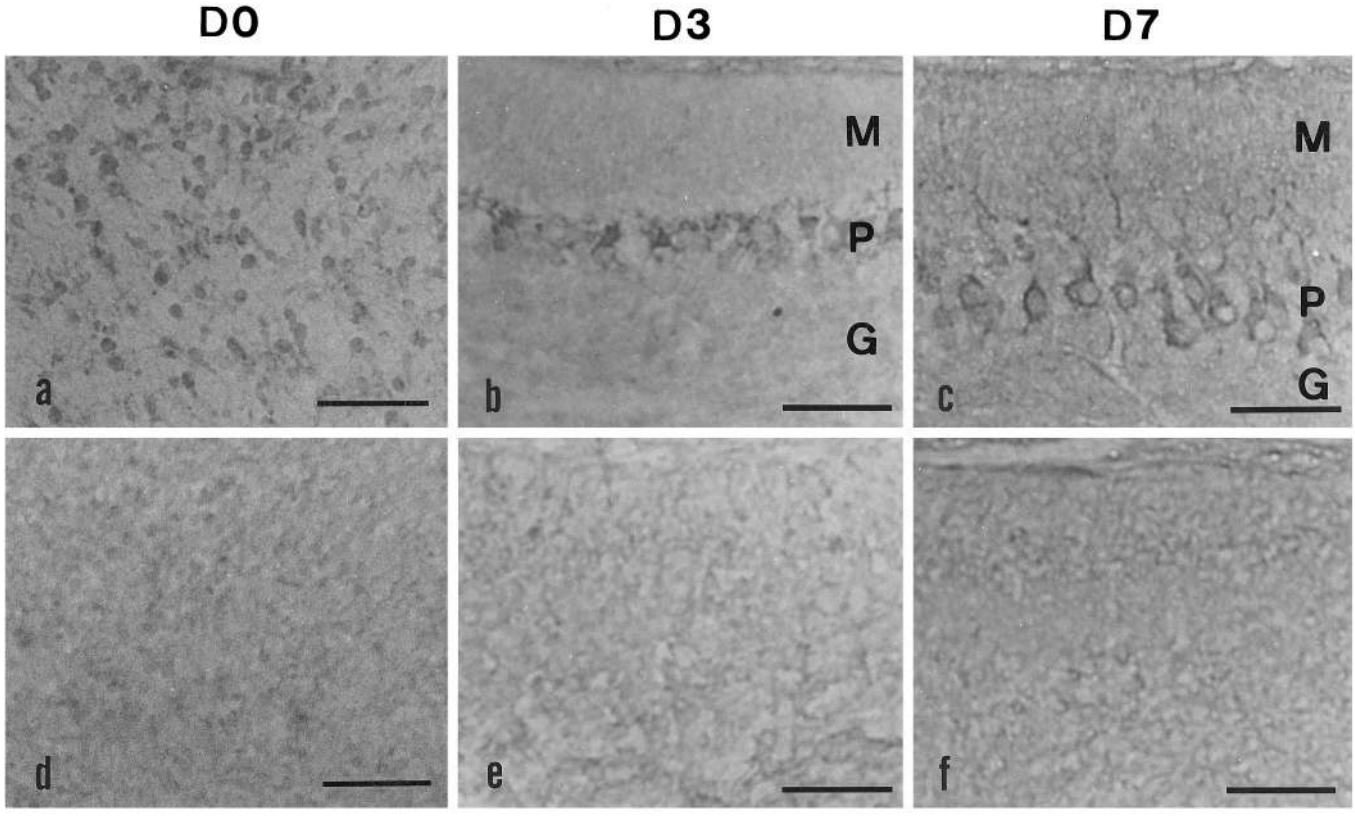

FIG. 5. Immunohistochemical staining with the antiserum to cytochrome P450scc (a-c) or with the antiserum preincubated with a saturating concentration of purified antigen (d-f) in the cerebellum of male rats at the ages of 0 day (D 0; a and d), 3 days (D 3; b and e) and 7 days (D 7; c and f). P, Purkinje cell layer; M, molecular layer; G, granular layer. *Bars* = 50 μm. The same result was obtained by repeated experiments using five animals at each age.

P450scc antigen in a saturating concentration (10 μg P450scc/ml) (Fig. 5d). At 3 days of age, P450scc-like immunoreactivity was observed only in somata of Purkinje cells that were located in a narrow zone between the molecular and granular layers (Fig. 5b). There was no immunoreaction with P450scc in either the molecular or granular layers at this age (Fig. 5b). At 7 days of age, a similar immunoreaction with P450scc was found not only in somata of Purkinje cells but also in the dendrites extending through the molecular layer (Fig. 5c). Preadsorbing the antibody with an excess amount of P450scc (10 μg/ml) also resulted in a complete absence of P450scc-like immunoreactivity in the Purkinje cell at the ages of 3 and 7 days (Fig. 5, e and f).

Immunohistochemical experiments were further carried out in the cerebellum of male rats at the ages of 7, 14, and 21 days to clarify changes in the P450scc-like immunoreaction in cerebellar Purkinje cells during the next neonatal period. Immunoreactive Purkinje cells developed and spread their dendrites into the molecular layer during this period (Fig. 6, a, c, and e). This change was associated with a decrease in the external granular layer (EGL) (Fig. 6, b, d, and f). The length of the immunopositive dendrite reached an adult level at 21 days of age, when the EGL almost disappeared (Fig. 6, e and f). Preadsorbing the antibody with P450scc (10 μg/ml) also resulted in a complete absence of P450scc-like immunoreactivity during 7–21 days (data not shown). In contrast to morphological changes in the immunoreactive Purkinje cell, there was no clear-cut difference in the intensity of the immunoreaction (Fig. 5, a, b, and c; Fig. 6, a, c, and e). On the other hand, no immunoreactivity for P450scc was observed in the nucleus of stained Purkinje cells (Fig. 5c; Fig. 6, a, c, and e).

Neonatal change in P450scc mRNA expression in the cerebellum

This experiment was designed to investigate neonatal changes in the expression of P450scc mRNA in the cerebellum. Total RNA was extracted from the cerebellum of male rats at the ages of 0, 3, 7, 14, 21, and 60 days and subjected to RT-PCR. As shown in Fig. 7, a single band corresponding to P450scc mRNA size, but not P450scc genomic DNA size, was detected at all of the observed ages. There was no clear-cut difference in the density of each band during neonatal development (Fig. 7), suggesting constant expression of the mRNA encoding for P450scc. Although the P450scc mRNA expression tended to decrease at 60 days of age, the change was not remarkable (Fig. 7). On the other hand, the ratio of initial total RNA amount used for RT-PCR reaction out of the total RNA extracted from one cerebellum was approximately 0.43 at 0 day, 0.31 at 3 days, 0.22 at 7 days, 0.09 at 14 days, 0.09 at 21 days, and 0.12 at 60 days. Therefore, the total P450scc mRNA level per whole cerebellum may increase at least during neonatal development.

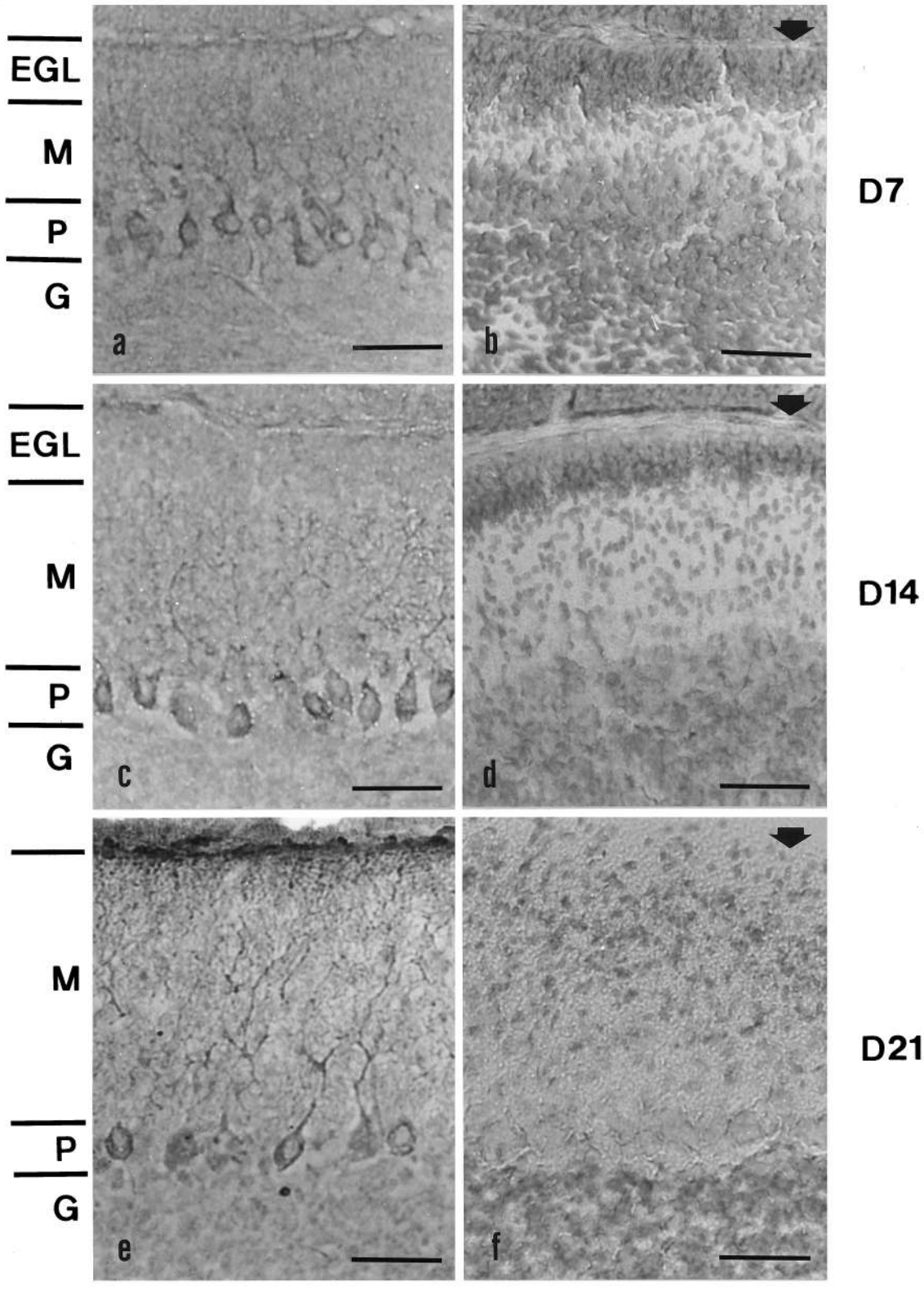

FIG. 6. Immunohistochemical staining with the antiserum to cytochrome P450scc (a, c, and e) in the cerebellar cortex of male rats at the ages of 7 days (D 7; a), 14 days (D 14; c), and 21 days (D 21; e). Histology of the cerebellar cortex was shown by Nissl staining (b, d, and f). All photographs are of the same magnification. P, Purkinje cell layer; M, molecular layer; G, granular layer; EGL, external granular layer in the cerebellar cortex. The molecular layer (M) increased from 7–21 days, while the EGL decreased during the same period and then disappeared at 21 days (b, d, and f). The *arrow* (b, d, and f) shows the border of one layer of the cerebellar cortex. *Bars* = 50 μm. The same result was obtained by repeated experiments using five animals at each age.

Neonatal change in pregnenolone in the cerebellum

Pregnenolone was measured in the cerebellum during neonatal development by a specific RIA using the antibody against pregnenolone. The pregnenolone concentration in the cerebellum was unexpectedly high at 0 day of age (8.52 ± 1.8 pmol/mg wet weight), decreased at 3 days of age, and reached a steady lower level from 7 days of age (0.68–1.20 pmol/mg; $P < 0.01$ *vs.*. 0 day; Fig. 8). The pregnenolone concentrations in plasma were significantly lower ($P < 0.05$, 0.01, or 0.001) than those in the cerebellum during neonatal development and in adulthood (Fig. 8). In addition to pregnenolone, pregnenolone sulfate ester was detected in both the cerebellum and plasma, but the level was low at all of the observed ages (Fig. 8).

On the other hand, the total amount of pregnenolone per whole cerebellum did not significantly change until 14 days of age (Fig. 9). However, the total cerebellar pregnenolone level increased significantly at 21 days of age ($P < 0.05$ *vs.*. 0 or 3 days; $P < 0.001$ *vs.*. 7 days) due to the increase in the cerebellar weight (Fig. 9). The amount of pregnenolone at 21 days of age was almost the same as that at 60 days of age (Fig. 9). In contrast, the total amount of pregnenolone sulfate ester in the cerebellum tended to increase during neonatal development, but the alteration was not significant (Fig. 9).

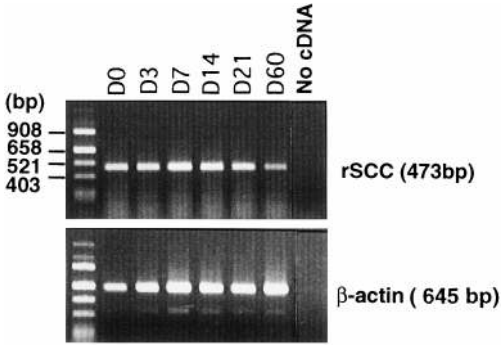

FIG. 7. RT-PCR analysis of P450scc mRNA in the rat cerebellum at the ages of 0, 3, 7, 14, 21, and 60 days. *Upper panel* shows a result of the gel electrophoresis of RT-PCR products for rat P450scc (rSCC). cDNA corresponding to 0.5 μg total RNA extracted from each cerebellar tissue was used for a PCR reaction and the 2/5 was applied on one lane. The lane labeled "No cDNA" was performed without template as the negative control. *Lower panel* shows a result of the RT-PCR for β-actin as the internal control, in which cDNA corresponding to 0.01 μg total RNA was used as template to avoid saturation of amplification. RT-PCR experiments were repeated four times using independently extracted RNA samples from different animals and indicated the same results.

Diurnal change in P450scc mRNA expression in the cerebellum

To examine diurnal changes in the expression of P450scc mRNA, male rats exposed to long day (LD) photoperiod (14-h light, 10-h dark) at 21 days of age were killed at 6 h (just lights on), 15 h (9 h after lights on), and 24 h (4 h after lights off). In this experiment, total RNA extracted from one animal was used as a sample, and RT-PCR analysis was performed on four different samples at each time following the same procedure of previous experiments. As shown in Fig. 10, there was no change in the expression of P450scc mRNA during a diurnal cycle.

Discussion

Employing immunohistochemical techniques, we first found the presence of cytochrome P450scc-like protein in the rat Purkinje cell, an important cerebellar neuron. Since preincubation of the antibody with purified P450scc antigen resulted in a complete disappearance of reaction product, the present immunohistochemical staining may be specific for P450scc. Such P450scc-immunoreactive cells were characterized immunohistochemically using the antibodies against marker proteins of Purkinje cells (IP_3 receptor) and glial cells (GFAP). The antibody against IP_3 receptor exactly recognized P450scc-immunoreactive cells, while the GFAP antibody failed to stain these cells. Therefore, we may conclude that P450scc-immunoreactive cells in the rat cerebellum are Purkinje neurons but not glial cells. Interestingly, a large number of granules were deeply stained in somata and dendrites of Purkinje cells. This result further suggests that P450scc-like protein is concentrated in some intracellular organelle, such as cytoplasmic mitochondria.

In the present study, Western immunoblot analysis with

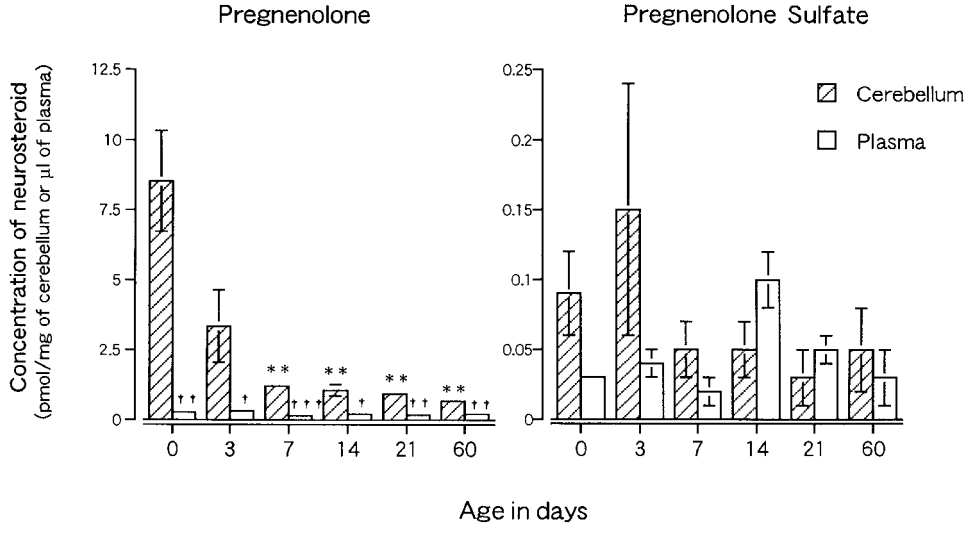

FIG. 8. Changes in the concentrations of pregnenolone and pregnenolone sulfate in the cerebellum and plasma in rats at the ages of 0, 3, 7, 14, 21, and 60 days. Each *column* and the *vertical line* represent the mean ± SEM (n = 4 samples). Significance of difference: **, $P < 0.01$ (*vs.* 0 day); †, $P < 0.05$, ††, $P < 0.01$, †††, $P < 0.001$ (*vs.* cerebellum).

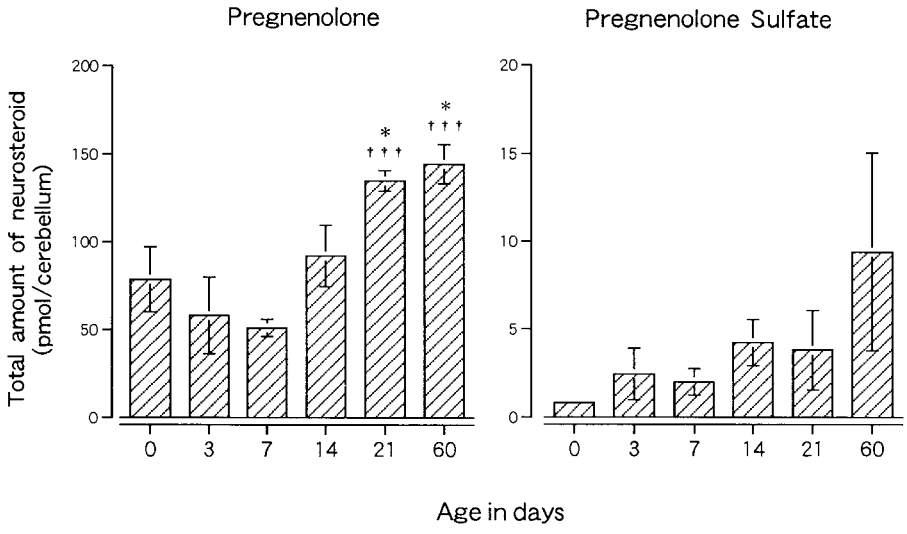

FIG. 9. Changes in the total amounts of pregnenolone and pregnenolone sulfate in the cerebellum in rats at the ages of 0, 3, 7, 14, 21, and 60 days. Each *column* and the *vertical line* represent the mean ± SEM (n = 4 samples). Significance of difference: *, $P < 0.05$ (*vs.* 0 day); †††, $P < 0.001$ (*vs.* 7 days).

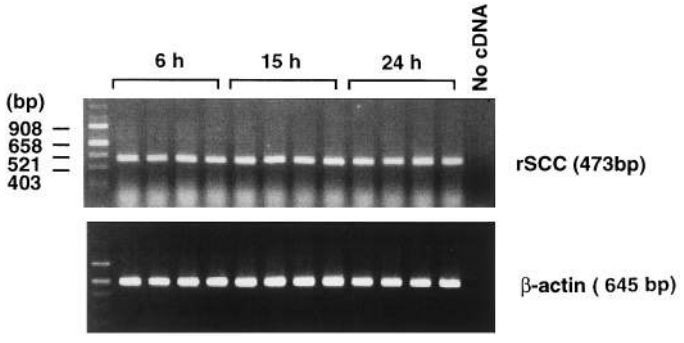

FIG. 10. RT-PCR analysis of P450scc mRNA during a diurnal cycle in the rat cerebellum. Cerebellar tissues were obtained at 6 h (just lights on, n = 4), 15 h (9 h after lights on, n = 4), and 24 h (4 h after lights off, n = 4). *Upper panel* shows a result of the gel electrophoresis of RT-PCR products for rat P450scc (rSCC). cDNA corresponding to 0.5 μg total RNA extracted from each cerebellar tissue was used for a PCR reaction and the 2/5 was applied on one lane. The lane labeled "No cDNA" was performed without template as the negative control. *Lower panel* shows a result of the RT-PCR for β-actin as the internal control, in which cDNA corresponding to 0.2 ng total RNA was used as template to avoid saturation of amplification.

the P450scc antibody confirmed the presence of P450scc-like protein in the rat cerebellum. In the cerebellum, the antibody predominantly recognized a protein band showing a similar electrophoretic mobility of testicular P450scc. RT-PCR analysis also indicated the expression of P450scc mRNA in the rat cerebellum. Therefore, it is possible that Purkinje cells possess P450scc, as P450scc-like immunoreactivity was restricted to this neuron in the cerebellum. However, the expressions of both P450scc-like protein and P450scc mRNA in the cerebellum seem to be lower than those in the testis.

It is well known that glial cells produce neurosteroids in the mammalian nervous systems (15–20). In contrast to glial cells, information on the neurosteroidogenesis in neurons is accumulating slowly in mammals. Some studies with the rat have indicated the neuronal P450scc expression in the nervous system (24, 35). However, to the best of our knowledge, whether neurons located in the mammalian brain produce neurosteroids remains unclear. Yamada and Ochi (48) previously suggested that some immunoreactive neurons were localized in various regions of the rat brain by immunohistochemical analysis with the anti-P450scc serum used in the present study. P450scc-immunoreactive cells were first identified as Purkinje cells in the rat by the present study. This finding is in agreement with our previous finding obtained by the avian species (12, 13). On the other hand, Sanne and Krueger (49) reported a lower expression of P450scc in the rat cerebellar granule layer and white matter. In addition, there is no report showing P450scc-like immunoreactivity in the Purkinje cell using other antisera against P450scc (14, 17, 19, 50). Therefore, to draw a firm conclusion concerning steroidogenesis in this neuron, further experiments with an independent antiserum are needed.

It has previously been reported that in rats the differentiation of Purkinje cells takes place at 3 days of age, when this neuron is located in a narrow zone between the molecular and granular layers (46, 47). Therefore, the question asked in the present study was when the steroidogenic enzyme P450scc appears in the Purkinje cell during cerebellar development. The present immunohistochemical analysis revealed a widespread distribution of the P450scc immunopositive cells throughout the cerebellum of infant male rats at 0 day of age. The immunoreaction examined at this age may be specific for P450scc, as it was inhibited by preincubation of the antibody with P450scc. It is therefore possible that the appearance of cytochrome P450scc occurs in the cerebellum before the differentiation of the first Purkinje cells in the rat. The next important question asked in the present study was to determine neonatal changes of P450scc localized in the Purkinje cell. We observed a steady immunoreaction with the P450scc antibody in soma of the Purkinje cell at 3 days of age, after which immunoreactive dendrites of the Purkinje cell extended into the developing molecular layer at advanced ages. It is well known that in rats the molecular layer is grown longer, as a consequence of the regression of the EGL from 10–21 days, and formation and cellular migration of the cerebellum is almost completed at 21 days (46, 47). In contrast to these morphological changes in the immunoreactive Purkinje cell, there was no clear difference in the intensity of its immunoreactivity during neonatal development. These immunohistochemical findings concur with the finding of the

present RT-PCR analysis showing a constant expression of the P450scc mRNA in the cerebellum during the neonatal period. As for widespread immunostaining at 0 day of age, the data obtained by RT-PCR analysis suggest that there is indeed P450scc expression in the cerebellum. The total P450scc mRNA level in the whole cerebellum may increase during neonatal development, due to the increase in the total amount of cerebellar RNA. Unlike neonatal life, the expression of P450scc mRNA might decrease slightly in adulthood, but the change was not remarkable.

In the present study, we further measured pregnenolone and its sulfate ester in the cerebellum as well as plasma during neonatal life. The pregnenolone concentration was much higher in the cerebellum than in plasma during neonatal development as well as in adulthood. Although we cannot rule out the possibility that pregnenolone produced in the peripheral steroidogenic glands accumulates in the cerebellum, these RIA results may reflect the presence of P450scc in the cerebellum. Pregnenolone concentrations in the cerebellum did not significantly change during the neonatal period except the rapid decrease just after birth. A similar decrease in the pregnenolone concentration in the whole brain has been reported in fetuses and newborn rats (5). A higher concentration of pregnenolone in the cerebellum of newborn rats is not consistent with the other results of the present study. Nonbrain sources of maternal and/or fetal pregnenolone might contribute to its accumulation in the cerebellum at this period. However, the profiles of intracerebellar changes in the concentration and the total amount of pregnenolone after 3 days of age, when the differentiation of the first Purkinje cells was completed, seem to be correlated with the results of immunohistochemistry and RT-PCR analysis. The total amount of pregnenolone in the cerebellum increased, due to the increase in the cerebellar weight, during 7–21 days of age, when immunoreactive Purkinje cells developed into the molecular layer without a significant change in the intensity of P450scc immunoreactivity. However, the conversion of pregnenolone to progesterone or dehydroepiandrosterone during neonatal life must be taken into account when studying the regulation of pregnenolone levels in the cerebellum. Therefore, more precise experiments, which measure the mRNAs encoding 3β-hydroxysteroid dehydrogenase and $P450_{17\alpha,lyase}$, are now in progress.

If pregnenolone and/or its sulfate ester produced in the Purkinje cell contribute to some physiological actions in the cerebellum, the P450scc expression would change under different physiological conditions. To test this hypothesis, therefore, we examined diurnal changes in the mRNA encoding P450scc in the cerebellum of neonatal rats exposed to long day (LD) photoperiod. However, we could not detect any clear-cut diurnal change in the P450scc mRNA expression. In contrast, there is evidence indicating a diurnal rhythm of the pregnenolone level in the rat whole brain (6, 8). Further studies are warranted to determine physiological changes in the expression of P450scc and the pregnenolone level in the cerebellum. On the other hand, it has been recently reported that pregnenolone and/or progesterone play a role in myelination but not axonal growth of the mouse glial cell in peripheral nervous system (51). Conversely, it has been suggested that in mice and rats neurosteroids may function as regulators of nerve growth (52). Therefore, the present finding indicating the presence of P450scc in the Purkinje cell may suggest functional roles of pregnenolone and/or its metabolites in promotion of the growth of neurons and/or glial cells in the cerebellum of neonatal rats.

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