PERGAMON

Journal of Steroid Biochemistry & Molecular Biology 85 (2003) 311–321

The Journal of Steroid Biochemistry & Molecular Biology

www.elsevier.com/locate/jsbmb

Biosynthesis and action of neurosteroids in the cerebellar Purkinje neuron☆

Kazuyoshi Tsutsui[a,b,*], Hirotaka Sakamoto[a,b], Kazuyoshi Ukena[a,b]

[a] *Laboratory of Brain Science, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima 739-8521, Japan*
[b] *Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Corporation, Tokyo 150-0002, Japan*

Abstract

The brain is considered to be a target site of peripheral steroid hormones. In contrast to this classical concept, new findings over the past decade have established that the brain itself also synthesizes steroids de novo from cholesterol through mechanisms at least partly independent of peripheral steroidogenic glands. Such steroids synthesized de novo in the brain, as well as other areas of the nervous system, are called neurosteroids. To understand neurosteroid actions in the brain, we need data on the specific synthesis in particular sites of the brain at particular times. Therefore, our studies for this exciting area of brain research have focused on the biosynthesis and action of neurosteroids in the identified neurosteroidogenic cells underlying important brain functions. We have demonstrated that the Purkinje cell, a typical cerebellar neuron, is a major site for neurosteroid formation in the brain. This is the first observation of neuronal neurosteroidogenesis in the brain. Subsequently, genomic and nongenomic actions of neurosteroids have become clear by a series of our studies using an excellent Purkinje cellular model. On the basis of these findings, we summarize the advances made in our understanding of biosynthesis and action of neurosteroids in the cerebellar Purkinje cell.

Keywords: Neurosteroids; Neuronal growth; Neurotransmission; Cerebellum; Purkinje cell

1. Introduction

Steroid hormones, supplied by the peripheral steroidogenic glands, regulate several important brain neuronal functions during development which persist into adulthood in vertebrates. Peripheral steroid hormones cross the blood–brain barriers, due to their chemically lipid solubility, and act on brain tissues through intracellular receptor-mediated mechanisms that regulate the transcription of specific genes [1–3]. Thus, a great deal was known about the brain as a target site of steroid hormones more than 10 years before. In contrast to this classical concept, new findings from several laboratories over the past decade have established unequivocally that the brain itself also synthesizes steroids de novo from cholesterol through mechanisms at least partly independent of peripheral steroidogenic glands (for reviews, see [4–8]). The pioneering discovery of Baulieu and his colleagues, using mammals, has opened the door of a new research field for many laboratories. Pregnenolone and dehydroepiandrosterone, as unconjugated steroids, and their fatty acid or sulfate esters, accumulate within the brains of several mammalian species [9–16]. The brain content of these steroids remains constant even after the removal of peripheral steroids by procedures such as adrenalectomy, castration, and hypophysectomy. This suggests that the mammalian brain can synthesize steroids de novo [9–11,13–15]. In contrast to mammals, little has been known regarding de novo steroidogenesis in the brain of nonmammals. We therefore looked for steroids, formed from cholesterol, in the brains of birds [17–25], amphibians [26] and fishes [27]. Independently, other groups, such as Vaudry's laboratory [28–30] and Schlinger's laboratory [31–33], also contributed to this area. The formation of several steroids from cholesterol is now known to occur in both mammalian and nonmammalian vertebrates. Such steroids synthesized in vertebrate brains are called neurosteroids.

To analyze neurosteroid actions in the brain, we need data on the specific synthesis of neurosteroids in particular sites of the brain at particular times. Such informations are essential to develop hypotheses predicting the potential roles of particular neurosteroids in the developing and adult brains. Thus, the studies for this exciting area of brain research should be focused on the biosynthesis and action of neurosteroids produced locally in the

☆ Presented at the 11th International Congress on Hormonal Steroids and Hormones and Cancer, ICHS & ICHC, Fukuoka, Japan, 21–25 October 2002.

* Corresponding author. Tel.: +81-824-24-6571; fax: +81-824-24-0759.
E-mail address: tsutsui@hiroshima-u.ac.jp (K. Tsutsui).

0960-0760/$ – see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0960-0760(03)00229-2

identified neurosteroidogenic cells underlying important brain functions. Pregnenolone, a 3β-hydroxy-Δ^5-steroid, is a main precursor of steroid hormones secreted by peripheral steroidogenic glands. The formation of pregnenolone is initiated by the cleavage of the cholesterol side-chain by cytochrome P450scc, a rate-limiting mitochondrial enzyme originally found in peripheral steroidogenic glands. It is therefore essential to demonstrate the formation of pregnenolone in particular cells of the brain. In the first immunocytochemical description of cytochrome P450scc by Le Goascogne et al. [34], an intense immunoreaction was detected in the white matter zone throughout the rat brain. The biochemical study in the rat further demonstrated that oligodendrocyte mitochondria convert cholesterol to pregnenolone [35]. The oligodendrocyte is a particular type of glial cell and produces the myelin of white matter. Thus, the expression and activity of P450scc in the glial cell have been established immunocytochemically and biochemically. In mammals, glial cells are now considered to play a major role in neurosteroid formation and metabolism in the brain and both oligodendrocytes and astrocytes are the primary site for pregnenolone synthesis [35–40]. This is also true for the presence of P450scc in glial cells located in the telencephalic and diencephalic regions of the quail [18,19] and the ring dove [41]. In contrast to glial cells, the concept of de novo neurosteroidogenesis in neurons in the brain has been uncertain. We have found the Purkinje cell, a typical cerebellar neuron, to be an active neurosteroidogenic cell, which possesses cytochrome P450scc and produces pregnenolone, in several vertebrate species (Fig. 1) [18–20,26,42]. We have further demonstrated that this neuron expresses several steroidogenic enzymes including P450scc and produces not only pregnenolone but also other neurosteroids, such as pregnenolone sulfate, progesterone and progesterone metabolites (Fig. 1) [18–20,23,26,27,42,43]. This is the first demonstration of neuronal de novo neurosteroidogenesis in the brain. Subsequently, neurosteroid actions have become clear by our studies using the Purkinje cell as an excellent cellular model, which is known to play an important role in the process of memory and learning.

This paper summarizes the advances made in our understanding of biosynthesis and action of neurosteroids in the cerebellar Purkinje cell. For detailed information of neurosteroids in glial cells the reader is referred to excellent reviews [4,7].

2. Materials and methods

2.1. Animals

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 14:10 h light–dark cycles (lights on at 6 a.m.) and were given food and tap water ad libitum. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the Hiroshima University, Japan.

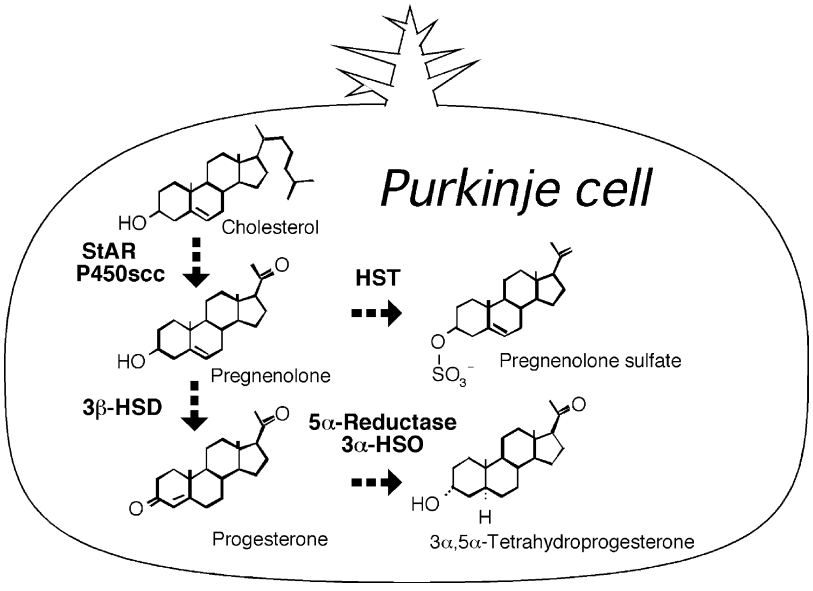

Fig. 1. Neurosteroidogenesis in the Purkinje cell. This neuron expresses several kinds of steroidogenic enzymes and produces pregnenolone, pregnenolone sulfate, progesterone and progesterone metabolites. The expression of P450scc remains during neonatal development and in adulthood, indicating the constant production of pregnenolone and its sulfate. This neuron also produces actively progesterone and 3α,5α-tetrahydroprogesterone, a progesterone metabolite, due to an increase of 3β-HSD activity, only during neonatal life. StAR, steroidogenic acute regulatory protein; P450scc, cytochrome P450 side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4-isomerase; HST, hydroxysteroid sulfotransferase; 3α-HSO, 3α-hydroxysteroid oxidoreductase.

2.2. Immunocytochemical analysis with P450scc antibody

Rats at various ages were deeply anesthetized with a chloroform, and then perfused transcardially with phosphate-buffered saline (PBS) followed by fixative solution [4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.3)]. 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 sunk. All cerebella were frozen-sectioned parasagittally at 40 μm thickness on a cryostat at −18 °C. The sections were processed according to the avidin–biotin–peroxidase complex (ABC) immunocytochemical technique with the floating method. 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 1:1000 for 36–48 h at 4 °C as described previously [42]. The anti-P450scc serum was raised in a rabbit using purified cytochrome P450scc from bovine adrenocortical mitochondria. The primary immunoreaction was followed by a 60 min-incubation with biotinylated anti-rabbit IgG (10 μg/ml) (Vector Laboratories, CA, USA) 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 (1:1000 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 Optical BH-2 microscope. To identify the cell type showing P450scc-like immunoreactivity, immunocytochemical analyses with three kinds of antibodies were subsequently performed as described previously [42]. 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, USA) was used in this study. A purified IgG fraction of the polyclonal rabbit antibody directed against the purified bovine GFAP (Dako Corp. Ltd., Glostrup, Denmark) was used as the GFAP antibody.

2.3. RT-PCR analyses of P450scc and 3β-HSD mRNAs

To determine expression of the mRNA encoding for rat P450scc in the cerebellum, RT-PCR analysis was performed using rats in adulthood and during neonatal development as described previously [42]. Total RNA of each cerebellum was isolated by the guanidinium thiocyanate–phenol–chloroform extraction method. Thirty micrograms of total RNA was reverse transcribed using Oligo dT primer and reverse-transcriptase 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 Oligo $dT_{12–18}$ (Pharmacia, Uppsala, Sweden), 15 U of ribonuclease inhibitor (Wako, Osaka, Japan), and 400 U of molony 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 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 dNTP mix, 12.5 pmol of each primer, and 1 U of *Taq* DNA polymerase (TOYOBO, Osaka, Japan). After denaturation at 95 °C for 3 min, the mixture was subjected to 30 thermal cycling in 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. 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. Oligonucleotides used as PCR primer and probe for mRNA detection, which were based on nucleotide sequences of rat P450scc [44] and rat β-actin [45], were as follows: P450scc sense primer 5′-TCAAAGCCAGCATCAAGGAG-3′ (nucleotides 1141–1160), P450scc antisense primer 5′-GCAGCCTGCAATTCATACAG-3′ (nucleotides 1594–1613), P450scc probe 5′-TTCTCAGGCATCAGGATGAG-3′ (nucleotides 1506–1525), β-actin sense primer 5′-GAGACCTTCAACACCCCAGC-3′ (nucleotides 2167–2186), and β-actin antisense primer 5′-CACAGAGTACTTGCGCTCAG-3′ (nucleotides 3004–3023).

To determine the expression of mRNA encoding for rat 3β-HSD in the cerebellum, RT-PCR analysis was also performed using rats during neonatal development and adults as described previously [43]. For PCR, an aliquot of the cDNA solution corresponding to 5 μg of the initial total RNA was used as a 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 dNTP mix, 12.5 pmol of each primer, and 1 U of *Taq* DNA polymerase (TOYOBO). After denaturation at 95 °C for 3 min, the mixture was subjected to 30 thermal cycling in a programmed temperature control system (PC700; ASTEC) 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. Each sample was electrophoresed through a 1.8% 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. Oligonucleotides used as PCR primer and probe for mRNA detection, based on nucleotide sequences of rat 3β-HSD [46] and rat β-actin [45], were as follows: 3β-HSD sense primer 5′-GCCCATGTACATTTATGGGG-3′ (nucleotides 729–748 of type I), 3β-HSD antisense primer 5′-CCCTTTCTGTCACTGAGACT-3′ (nucleotides 1302–1283 of type I), 3β-HSD probe 5′-TTTTCTGCTTGGCTTCCTCC-3′ (nucleotides 1228–1209 of type I), β-actin sense primer 5′-GAGACCTTCAACACCCCAGC-3′ (nucleotides 2167–2186), and β-actin antisense primer 5′-CACAGAGTACTTGCGCTCAG-3′ (nucleotides 3023–3004). It has been reported that rat 3β-HSD has four different isoforms (types I–IV) [46–48]. In this study, the 3β-HSD sense and antisense primers, which are same and complementary to a common sequence of types I and II, give a 574 bp amplified fragment of 3β-HSD (isoform types I and II) gene.

2.4. Biochemical analysis of 3β-HSD activity

To examine 3β-HSD activity in the cerebellum, biochemical analysis was performed using cerebellar slices as described previously [43]. The cerebellar slices were incubated at 37 °C for 5 or 15 min in 0.5 ml physiological saline containing 1,000,000 cpm of [7-^{3}H] pregnenolone (specific activity, 19.9 Ci/mmol; New England Nuclear, Boston, MA, USA) and 4% propylene glycol. The incubation medium was constantly gassed with 95% O_2 and 5% CO_2. At the end of the incubation period, 2 ml ethyl acetate were added, and the slices were homogenized with a glass–glass homogenizer. After stirring the homogenate for 15 min followed by centrifugation at 3000 × *g* for 5 min, the organic phase was removed. This extraction step was repeated twice. The combined organic extracts were dried down, redissolved in 70% acetonitrile (ACN), and filtrated through a membrane filter (0.45 μm pore size; Ultrafree-MC; Millipore Corp., Bedford, MA, USA). To detect labeled steroids formed from [7-^{3}H] pregnenolone, filtrates were subjected to HPLC analysis using a reversed-phase column, LiCrospher 100 RP-18 (4.0 mm × 250 mm; Kanto Chemical Corp. Inc., Tokyo, Japan). The column was eluted with an isocratic elution of 70% ACN at a flow rate of 0.7 ml/min. The eluate was fractionated every 0.5 min from 10 to 20 min and counted in a liquid scintillation counter. Reference standards of tritiated pregnenolone and progesterone were chromatographed under conditions similar to those used for the tissue extracts and were detected using a liquid scintillation counter. To confirm the involvement of 3β-HSD activity in the formation of the radioactive peak of progesterone, cerebellar slices were incubated with saline containing trilostane (Mochida, Tokyo, Japan), a specific inhibitor of 3β-HSD, and subjected to HPLC analysis in a manner similar to that described above.

2.5. In situ hybridization of 3β-HSD mRNA

The site of 3β-HSD expression in the cerebellum was localized by in situ hybridization. Rats were deeply anesthetized with chloroform before transcardial perfusion with PBS followed by fixative solution (4% paraformaldehyde in PBS). Six microns parasagittal sections of the cerebella were made using a cryostat at −18 °C and were placed onto 3-aminopropyltriethoxysilane-coated slides. In situ hybridization was carried out as described previously [43]. In brief, the fixed sections were rehydrated with PBS and treated with 0.2 N HCl for 20 min, followed by 1 μg/ml proteinase-K at 37 °C for 10 min. After postfixation with 4% paraformaldehyde in PBS for 5 min, the slides were kept in 40% deionized formamide in 4× SSC (1× SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0) for 30 min. Hybridization was carried out at 37 °C for 15–17 h with 50 ng/ml digoxigenin–oligonucleotide probe mixture dissolved in the hybridization medium containing 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 0.6 M NaCl, 10% dextran sulfate, 1× Denhardt's solution, 250 μg/ml yeast tRNA, 125 μg/ml salmon sperm DNA, and 40% deionized formamide. The sections were treated with 1.5% blocking reagent (Boehringer Mannheim, Vienna, Austria) in PBS and incubated with alkaline phosphatase-labeled sheep antidigoxigenin antibody (1:1000 dilution in the blocking solution, Boehringer Mannheim) for 1 h. Immunoreactive products were detected by immersing the sections for 48 h in a substrate solution (0.035% nitro blue tetrazolium and 0.018% 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris–HCl, 100 mM NaCl, and 50 mM $MgCl_2$, pH 9.5), and the expression of 3β-HSD mRNA was observed using an Olympus Optical BH-2 microscope.

Oligonucleotides used as the 3β-HSD antisense probe mixture were as follows: 5′-TCCAGCAGGAAGGCAAGCCAGTAGAGCAGGGGCAGAGGAAGGCTCC-3′ [nucleotides 1022–1067 of 3β-HSD type I, which are complementary to a common sequence of four different isoforms (types I–IV) of 3β-HSD] and 5′-TGTCTCCCTGTGCTGCTCCACTAGTGTCCCGATCCACTCCGAGGT-3′ [nucleotides 1228–1272 of 3β-HSD type I, which are complementary to a common sequence of four different isoforms (types I–IV) of 3β-HSD except for one base differing from type III]. Control for specificity of the in situ hybridization of 3β-HSD mRNA was performed by the addition of an excess amount (4000-fold) of homologous or nonhomologous unlabeled

oligonucleotides to the hybridization medium applied to the sections. β-actin antisense primer used in the RT-PCR analysis was employed as a nonhomologous competitor.

2.6. Radioimmunoassays of pregnenolone, pregnenolone sulfate and progesterone

To measure levels of pregnenolone, pregnenolone sulfate and progesterone in the cerebellum during neonatal development and in adulthood, cerebellar extracts were applied to the radioimmunoassays (RIAs) of pregnenolone, pregnenolone sulfate and progesterone as described previously [42,43]. Cerebella were homogenized in 5 ml ice-cold PBS (pH 7.6) with a Teflon glass homogenizer and applied to steroid extraction. Extraction of unconjugated steroids or steroid sulfates was performed according to the previous method [42,43]. To measure the concentrations of pregnenolone and its sulfate, aliquots from both extracts of organic (pregnenolone) and water (pregnenolone sulfate) phases were applied to the RIAs using the antiserum to pregnenolone (Radioassay Systems Laboratories Inc., Immuchem Corp., Carson, CA, USA) and [7-^{3}H] pregnenolone (specific activity, 23.5 Ci/mmol; New England Nuclear) [42]. The antiserum 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, USA). The least detectable amount was 0.1 ng/ml, and intraassay variation was less than 7%.

To measure the progesterone concentration, aliquots of organic extracts were assayed in a progesterone RIA using the antiserum to progesterone (Scantibodies Laboratories Inc., Santee, CA) and [1, 2, 6, 7-^{3}H] progesterone (specific activity, 115 Ci/mmol; New England Nuclear) [43]. The antiserum cross-reacted with deoxycorticosterone at 3.3%, 17α-hydroxyprogesterone at 0.6%, and pregnenolone at less than 0.1%. Separation of bound and free steroid was performed by centrifugation after reaction with the IgG SORB (The Enzyme Center Inc.). The least detectable amount was 0.1 ng/ml, and intraassay variation was estimated as less than 7%.

2.7. Slice culture of cerebella and in vitro steroid treatment

Cerebella of pups at 5 days of age were dissected out into ice-cold Hank's balanced salt solution (HBSS; pH 7.3), and embedded in 2% low-gelling temperature (30–31 °C) agar in HBSS at 35–38 °C as described previously [49,50]. Immediately after embedding, vermal parasagittal slices (400 μm thick) were cut on a microslicer and cultured on a porous membrane (Intercell TP, Kurabo, Osaka, Japan) which was floated at the interface between air and a culture medium. The culture medium was composed of a 1:1 mixture of Dulbecco's modified essential medium and Ham's F-12 (Sigma), supplemented with insulin (5 μg/ml; Sigma), apo-transferrin (100 μg/ml; Sigma), putrescine (100 μM; Sigma), sodium selenite (30 nM), D-glucose (6 mg/ml), penicillin G potassium (100 U/ml), and streptomycin sulfate (100 μg/ml). In this study, the culture medium contained 5% fetal bovine serum ((v/v); Sanko, Tokyo, Japan) for the first 2 days of culture (2 DIV). Cultures were maintained at 37 °C in an atmosphere of humidified 95% air and 5% CO_2.

To investigate morphological changes of Purkinje cells induced by progesterone or 3α,5α-tetrahydroprogesterone, a progesterone metabolite, these neurosteroids were applied to granuloprival cerebellar cultures for 3 days after 2 DIV, and cultures were fixed at 5 DIV as described previously [49,50]. Crystalline progesterone or 3α,5α-tetrahydroprogesterone (Sigma) was dissolved into absolute ethanol and applied to the culture medium at various concentrations (progesterone: 0.1, 1, 10, 100 nM and 1 μM; 3α,5α-tetrahydroprogesterone: 1, 10 and 100 nM). The effect of an anti-progestin, RU 486 [mifepristone; 17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl) estra-4,9-dien-3-one-6-7] (Biomol, Plymouth Meeting, PA, USA), was examined at a concentration of 1 μM in a progesterone-treated (10 nM) group. RU 486 alone was also tested as a control. All cultures were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde and 15% saturated picric acid (v/v) in PB (pH 7.3) overnight at 4 °C and subjected to immunocytochemical labeling of Purkinje cells using a calcium-binding protein (calbindin) antibody, followed by the morphological analysis of Purkinje cells.

2.8. In vivo steroid treatment

Progesterone dissolved in sesame oil (50 μg/25 μl) was injected into the cerebrospinal fluid around the posterior vermis of pups once per day for 4 days from 3 days of age [49,50]. Pups receiving injections of the vehicle alone (sesame oil) served as controls. At 7 days of age, pups were deeply anesthetized with chloroform before transcardial perfusion with PBS, followed by fixative solution [2% paraformaldehyde, 2.5% glutaraldehyde and 15% saturated picric acid (v/v) in PB]. Vermal cerebella were dissected out and sectioned parasagittally at 50 μm thickness with a microslicer before immunostaining with calbindin antibody.

2.9. Immunocytochemical labeling of Purkinje cells with calbindin

Purkinje cells were identified by immunostaining with a mouse monoclonal antibody raised against a calcium-binding protein, calbindin-D_{28k} (Sigma). Cerebellar sections and slice cultures were processed for immunocytochemistry. After elimination of endogenous peroxidase activity with 3% H_2O_2 (0% for electron microscopic study) and blocking nonspecific-binding components with 1% normal horse serum and 1% BSA, the sections and slice cultures were immersed overnight at 4 °C with the monoclonal antibody

against calbindin at a dilution of 1:50,000. Immunoreactive products were detected with an avidin–biotin kit (Vectastain Elite Kit, Vector Laboratories) followed by DAB reaction.

2.10. Light microscopic analysis of the morphology of Purkinje cells

After immunostaining for calbindin, Purkinje cells with dendrites and axons visible were randomly selected in each vermal lobe of slice cultures derived from in vitro studies [49]. Purkinje cells were randomly selected in the vermal lobe IX around the site of in vivo steroid injection. The selected Purkinje cell was traced with a camera lucida drawing tube, and these drawings were converted to digital files using a scanner. The whole area, cross-sectional soma area and perimeter of Purkinje cells were measured from these camera lucida drawings in each selected calbindin-immunostained lobe using an NIH-Image software package. To measure the dendritic area of Purkinje cells, cross-sectional soma area was deducted from whole area of each cell.

To analyze the effects of progesterone on Purkinje dendritic morphology, the number of dendritic spine per unit length of dendrite and total dendritic length per cell of Purkinje cells were further measured. Camera lucida reconstructions were made as stick figures ("skeletonized" drawings), representing the exact length and complexity of the dendritic arbor. Purkinje cells with dendrites and axons visible were randomly selected in each vermal lobe of slice cultures. These reconstructions were converted to digital files using a scanner. Total dendritic lengths of Purkinje cells were determined with an NIH-Image software package. For each dendritic segment selected, spine density was measured as follows; the selected segment was traced with a camera lucida drawing tube; all the dendritic spines visible along that segment were counted; the length of each segment was also measured from its camera lucida drawing with an NIH-Image software package; and data were then expressed as the number of spines per unit length of dendrite. Differences in the dendritic spine number and total dendritic length of Purkinje cells following treatment with vehicle or progesterone were analyzed. To verify the action of endogenous progesterone on Purkinje dendritic outgrowth, pups injected with the anti-progestin, RU 486 were further used for morphological analysis [49,50].

2.11. Electron microscopic analysis of the morphology of Purkinje cells

For electron microscopy, calbindin-immunocytochemically stained lobe sections of progesterone- and vehicle-treated pups at 7 days of age were postfixed in 1% osmium tetroxide in PB, dehydrated in ascending grades of ethanol, and then embedded flat in epoxy resin (Quetol-812, Nisshin EM, Tokyo, Japan) as described previously [49,50]. Ultrathin sections (60 nm in thickness) containing calbindin-immunoreactive Purkinje dendrites in lobe IX were collected in slot grids coated with Formvar film, electron-stained with uranyl acetate and lead citrate, and viewed under an H-600A electron microscope (Hitachi, Tokyo, Japan). Electron microphotographs of random regions in the molecular layer of lobe IX were generated from different animals per each experimental group. The number of asymmetrical synapses having a postsynaptic density was counted and the density of synapses per volume was calculated [49].

2.12. Immunocytochemistry of progesterone receptor

Vermal cerebella were removed, stored in fixative overnight (3.75% acrolein and 2% paraformaldehyde in PB), and then soaked in a refrigerated sucrose solution (30% sucrose in PB) until they sank. All cerebella were frozen-sectioned parasagittally at 40 μm thickness on a cryostat at −18 °C. The free-floating sections were first treated with 1% $NaBH_4$ for 10 min, followed by 1% BSA, 3.3% normal goat serum, and 0.1% Triton X-100 in PBS for 1 h. They were then immersed for 72 h at 4 °C with a polyclonal rabbit antiserum (1:1000) directed against the DNA-binding domain of the human progesterone receptor (PR) (amino acids 533–547, GLPQVYPPYLNYLRP; Dako). The antiserum cross-reacted with both isoforms (types A and B), and its specificity has been described previously [51–54]. Immunoreactive products were detected with an ABC kit (Vectastain Elite Kit, Vector Laboratories), followed by DAB reaction. Control procedures consisted of (1) preadsorbing the working dilution of the primary antiserum with a saturating concentration of the synthetic peptide corresponding to the DNA-binding domain (amino acids 533–547) of human PR (100 μg/ml), and (2) substituting normal rabbit serum for the primary antiserum at a dilution of 1:1000. The sections were incubated with these control sera in a similar way as with the PR antiserum and studied using an Olympus Optical BH-2 microscope.

3. Results and discussion

3.1. Neurosteroidogenesis in Purkinje cells

In our immunocytochemical studies with the quail brain using an antibody against cytochrome P450scc, the striking observation was the distribution of immunoreactive cells in the cerebellar cortex [18–20]. The distribution of immunoreactive cell bodies and fibers in the cerebellar cortex was coincident with the location of somata and dendrites of Purkinje cells [18–20]. Western immunoblot analysis confirmed the presence of P450scc in Purkinje cells [18]. These findings obtained in the avian brain have provided the first evidence for the location of cytochrome P450scc in neurons in the brain, because the Purkinje cell is a typical cerebellar neuron. Whether neurons in the brain of other vertebrate species possess cytochrome P450scc and produce pregnenolone and

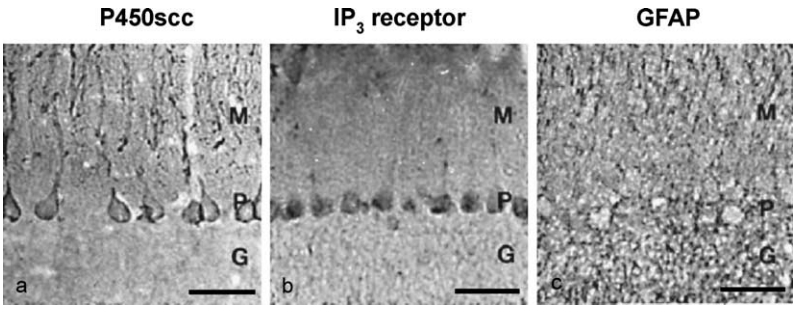

Fig. 2. Immunocytochemical staining with the antiserum to P450scc, IP_3 receptor, or GFAP in the adult cerebellum. The antiserum to IP_3 receptor or to GFAP was used as a specific marker of Purkinje cells or glial cells. P450scc, cytochrome P450 side-chain cleavage enzyme; IP_3 receptor, inositol triphosphate receptor; GFAP, glial fibrillary acidic protein; P, Purkinje cell layer; M, molecular layer; G, granular layer. Scale bars: 50 μm.

its sulfate ester still remained unclear. We therefore investigated the presence of P450scc in the Purkinje cell using rats [42]. As shown in Fig. 2, an 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. Thus, immunoreaction with P450scc was confined to the somata and dendrites of Purkinje cells in the rat cerebellum [42]. In addition, the expression of both P450scc protein and P450scc mRNA was detected in the rat cerebellum [42]. Interestingly, P450scc appeared in the rat Purkinje cell immediately after its differentiation and the expression of this enzyme persisted during neonatal development into adulthood, indicating the constant production of pregnenolone and its sulfate ester [42]. In addition to higher vertebrates, our recent studies with amphibians further identified P450scc in the Purkinje cell of *Xenopus laevis* and *Rana nigromaculata* [26]. Altogether, these findings obtained in both higher and lower vertebrates [18–20,26,42] indicate that Purkinje cells possess P450scc and produce pregnenolone and its sulfate ester (Fig. 1). On the other hand, the steroidogenic acute regulatory protein (StAR) was also found in Purkinje cells [55]. StAR is involved in the transport of cholesterol to the inner mitochondrial membrane, in which P450scc is localized, and thus plays a key role in steroid biosynthesis in peripheral steroidogenic glands [56,57]. StAR may also contribute to the regulation of pregnenolone formation in the Purkinje cell (Fig. 1).

Subsequently, we have extended our understanding of the expression of 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4-isomerase (3β-HSD) in the Purkinje cell. The progesterone formation is performed by 3β-HSD which catalyzes the dehydrogenation and isomerization of 3β-hydroxy-Δ^5-steroids (pregnenolone and dehydroepiandrosterone) into Δ^4-ketosteroids (progesterone and androstenedione, respectively) and is expressed in peripheral steroidogenic glands [58]. RT-PCR and biochemical analyses showed the expression of 3β-HSD and its enzymatic activity in the rat cerebellum [43]. Employing in situ hybridization of 3β-HSD mRNA, the site of 3β-HSD expression was localized in Purkinje cells and external granule cells [43]. Thus, both P450scc and 3β-HSD are expressed in Purkinje cells (Fig. 1). Surprisingly, the expression of 3β-HSD increased during neonatal life, unlike P450scc (Fig. 3). Such an age-dependent expression of 3β-HSD was confirmed by biochemical studies together with HPLC analysis, indicating an increase of progesterone formation during neonatal life (Fig. 4). Recently, we also found the progesterone metabolite 3α,5α-tetrahydroprogesterone in the neonatal rat cerebellum [59,60]. The expression of 3β-HSD in Purkinje cells was also evident in other vertebrate species [5,27].

Altogether, the Purkinje cell is an important neurosteroidogenic cell in the brain (Fig. 1) (for reviews, see [5,6,61,62]). Interestingly, the Purkinje cell produces constantly pregnenolone and its sulfate ester during neonatal development and in adulthood (Fig. 1). Furthermore, this neuron actively produces progesterone and 3α,5α-tetrahydroprogesterone as products of an increase of 3β-HSD activity during neonatal life (Fig. 1). Thus, our studies have

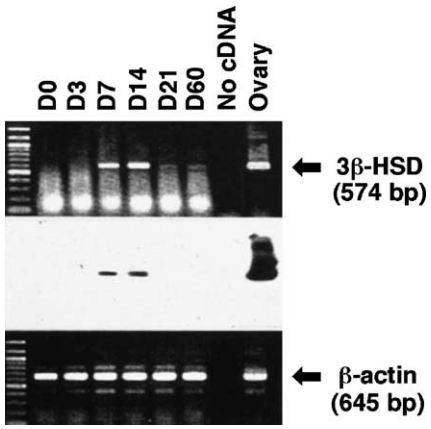

Fig. 3. RT-PCR analysis of 3β-HSD mRNA in the cerebellum during neonatal development. The upper panel shows a result of gel electrophoresis of RT-PCR products for rat 3β-HSD, and the middle panel shows an identification of the band by Southern hybridization using digoxigenin-labeled oligonucleotide probe for rat 3β-HSD. The ovary was used as a positive control tissue. The lane labeled No cDNA was performed without template as the negative control. The lower panel shows a result of the RT-PCR for β-actin as the internal control. 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4-isomerase.

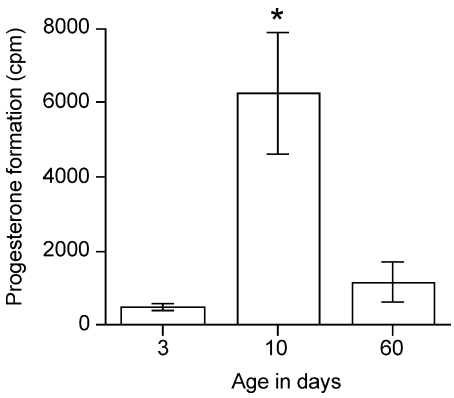

Fig. 4. Comparison of progesterone formation among cerebella at various ages. Cerebellar slices adjusted to the same weight were incubated with tritiated pregnenolone for 15 min and subjected to HPLC analysis. Each column and error bar represent the mean ± S.E.M. radioactivity corresponding to progesterone ($n = 4$ samples in each group). $^{*}P < 0.05$ (vs. 3 and 60 days by ANOVA, followed by Duncan's multiple range test).

provided the opportunity to understand neuronal neurosteroidogenesis in the brain (Fig. 1).

3.2. Genomic actions of neurosteroids on neuronal growth, spinogenesis and synaptogenesis in Purkinje cells

To analyze neurosteroid actions in the brain, we need data on the specific synthesis in particular sites of the brain at particular times. The Purkinje cell should serve as an excellent cellular model for the study of neurosteroid actions, because this neuron produces several kinds of neurosteroids at particular times (Fig. 1) (for reviews, see [5,6,61,62]). As mentioned above, Purkinje cells actively synthesize progesterone and 3α,5α-tetrahydroprogesterone, a progesterone metabolite, during the neonatal period, as the expression of 3β-HSD and its enzymatic activity increase in neonatal rats (Figs. 3 and 4) [43]. With these findings as a background, we examined the effects of progesterone and 3α,5α-tetrahydroprogesterone, produced as neurosteroids in the Purkinje cell only during neonatal life, on neuronal growth, spinogenesis and synaptogenesis in the rat cerebellum [49,50]. Interestingly, in vitro studies using cultured cerebellar slices of newborn rats showed that progesterone promotes dendritic growth (Fig. 5) and dendritic spine formation (Fig. 6) of the Purkinje cell. A similar result was obtained by in vivo studies [49,50]. Electron microscopic analysis further revealed that progesterone induces an increase of the density of synapses on the Purkinje cell (Fig. 7). Furthermore, intranuclear receptors for progesterone were expressed in the Purkinje cell during neonatal life (Fig. 8). In contrast to progesterone, we could not detect any significant effect of 3α,5α-tetrahydroprogesterone on Purkinje development [49,50]. These results indicate that progesterone promotes the dendritic growth, spinogenesis, and synaptogenesis of Purkinje cells by genomic mechanisms (Fig. 9).

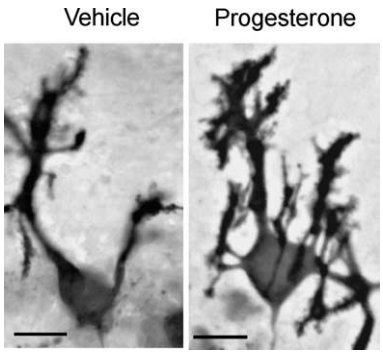

Fig. 5. Effect of progesterone on the dendritic growth of Purkinje cells. Cerebellar cultures from newborn rats grown for 5 DIV and immunostained for calbindin. Purkinje cell dendrites in the progesterone-treated group appeared to be well-developed compared with the control group. Scale bars: 20 μm.

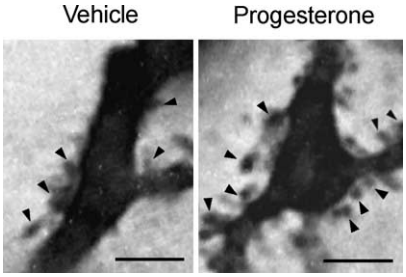

Fig. 6. Effect of progesterone on the density of Purkinje cell dendritic spines. Cerebellar cultures from newborn rats grown for 5 DIV and immunostained for calbindin. Progesterone treatment induced the greater density of dendritic spines of the Purkinje cell. Arrowheads indicate spine structures. Scale bars: 5 μm.

It is well known that in the rat marked morphological changes occur in the cerebellum after birth during neonatal life. According to Altman [63,64], rat Purkinje cells differentiate just after birth, and the formation of the cerebellar cortex becomes complete in the neonate, through the

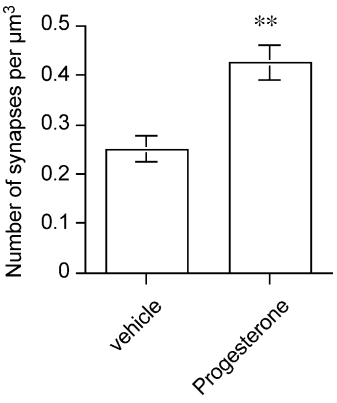

Fig. 7. Effect of progesterone on the density of axospinous synapses on Purkinje cells. Quantitative electron microscopic analysis. Progesterone administration to newborn rats increased the axospinous synapse density of Purkinje cells. Each column and error bar represent the mean ± S.E.M. ($n = 6$ animals in each group). $^{**}P < 0.01$ (by Student's t-test).

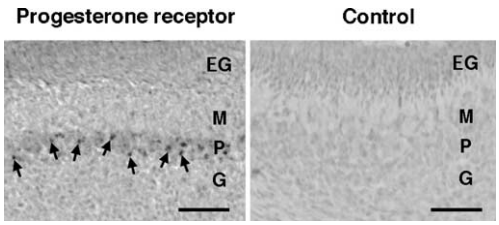

Fig. 8. Immunocytochemical staining with the antiserum to progesterone receptor in the neonatal cerebellum. Arrows indicate Purkinje cells expressing progesterone receptor. Preadsorbing the antiserum with an excess amount of the synthetic progesterone receptor peptide used as antigen (DNA-binding domain) resulted in a complete absence of immunoreactivity in Purkinje cells. EG, external granular layer; M, molecular layer; P, Purkinje cell layer; G, internal granular layer. Scale bars: 50 μm.

processes of migration of external granular cells, neuronal and glial growth, and synaptogenesis. Thus, postnatal development in the cerebellum is dramatic during neonatal life, when the formation of progesterone in the cerebellum is high [43,59,60]. Accordingly, genomic actions of progesterone may be involved in the formation of the cerebellar neuronal circuit that occurs during neonatal life by promoting neuronal growth and neuronal synaptic contact.

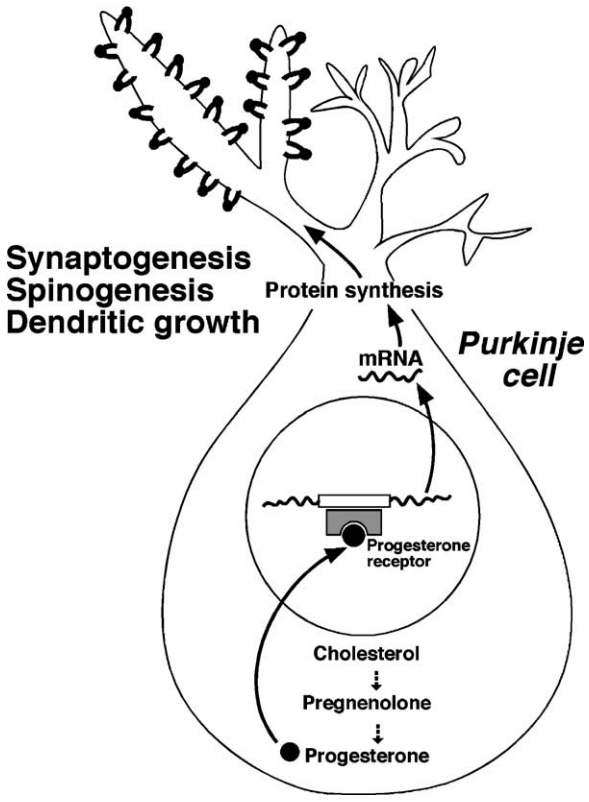

Fig. 9. Genomic action of progesterone produced in the Purkinje cell during neonatal development. Progesterone acts on the Purkinje cell through intracellular receptor-mediated mechanisms that promote dendritic growth, spinogenesis, and synaptogenesis in this neuron by genomic mechanisms. Such genomic actions of progesterone may contribute to the formation of the cerebellar neuronal circuit during neonatal life.

3.3. Nongenomic actions of neurosteroids on GABAergic neurotransmission in Purkinje cells

Until recently, we believed that all steroid hormones regulate biological functions by genomic mechanisms. The genomic action of steroid hormones presumes that steroid hormones cross the plasma membrane and bind to and activate specific intracellular steroid receptors. The activated steroid receptors modulate gene transcription and protein synthesis. In addition to genomic actions, we also focused on nongenomic actions of neurosteroids in the Purkinje cell and indicated that pregnenolone sulfate contributes to important events in the cerebellum by nongenomic mechanisms. To understand the mode of action of neurosteroids, produced in Purkinje cells, we examined the effects of pregnenolone and its sulfate ester on synaptic currents in Purkinje cells of the rat [6,19,59–61]. Inhibitory postsynaptic currents (IPSCs) in Purkinje cells were recorded in a cerebellar slice by the patch-clamp method. Pregnenolone sulfate increased, in a dose-related way, the frequency of IPSCs within 1 min of perfusion, indicating that this effect is unlikely to be induced via gene transcription. In contrast, pregnenolone had no effect on the frequency of IPSCs. The IPSCs recorded in the Purkinje cells were completely blocked by bicuculline, a γ-aminobutyric acid A ($GABA_A$) receptor antagonist, suggesting that they are mediated by $GABA_A$ receptors. Thus, pregnenolone sulfate, produced in Purkinje cells, may modulate GABAergic transmission by nongenomic actions on GABAergic neurons rather than by genomic mechanisms (Fig. 10). In addition to these findings in the cerebellum, new findings have been obtained in other brain regions that some neurosteroids, such as pregnenolone, pregnenolone sulfate, progesterone and 3α,5α-tetrahydroprogesterone, may

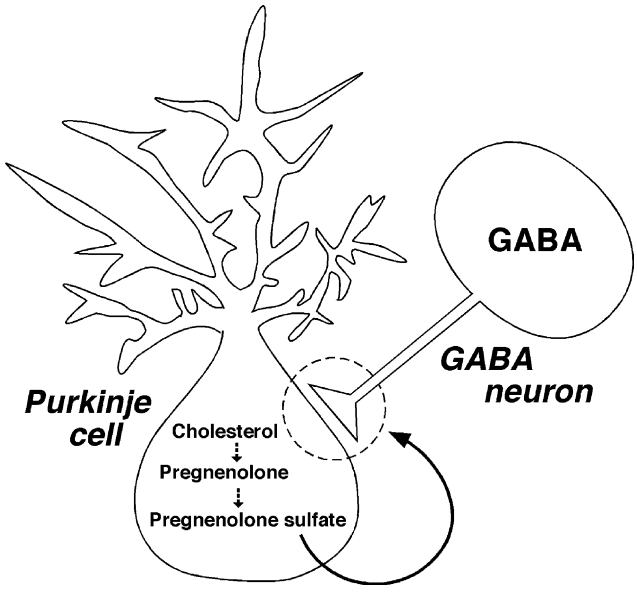

Fig. 10. Nongenomic action of pregnenolone sulfate produced in the Purkinje cell. Pregnenolone sulfate modulates GABAergic transmission by means of the nongenomic action on GABAergic neurons.

mediate their actions through ion-gated channel receptors rather than by genomic mechanisms (for reviews, see [4–8]).

4. Conclusions

De novo steroidogenesis from cholesterol is a conserved property of vertebrate brains, and such steroids synthesized de novo in the brain are called neurosteroids. Our studies gave the opportunity to understand the biosynthesis and action of neurosteroids in the Purkinje cell, a major site of neurosteroidogenesis in the brain. The Purkinje cell served as an excellent cellular model for understanding the plasticity of cerebellar neurons in relation to actions of neurosteroids. Because Purkinje cells play an important role in the process of memory and learning, future attention should be focused on behavioral studies, as well as morphological and electrophysiological studies using steroidogenic enzyme and/or neurosteroid receptor knock-out animals.

Acknowledgements

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, and Culture, Japan (11170237, 11354010, 12440233, 12894021 and 13210101 to K.T.) and the Narishige Zoological Science Award (to K.U.). H.S. is supported by a Research Fellowship of the Japan Society for the Promotion of Science (JSPS) for Young Scientists. We are grateful to Drs. Y. Furukawa, M. Takase, and M. Usui (Hiroshima University, Higashi-Hiroshima, Japan) for their collaboration.

References

[1] K. Fuxe, J.A. Gustafsson, L. Wetterberg, Steroid Hormone Regulation of the Brain, Pergamon Press, Oxford, 1981.
[2] A.P. Arnold, R.A. Gorski, Gonadal steroid induction of structural sex differences in the CNS, Annu. Rev. Neurosci. 7 (1984) 413–442.
[3] B.S. McEwen, Steroid hormones are multifunctional messengers in the brain, Trends Endocrinol. Metab. 2 (1991) 62–67.
[4] E.E. Baulieu, Neurosteroids: of the nervous system, by the nervous system, for the nervous system (review), Rec. Prog. Hormone Res. 52 (1997) 1–32.
[5] K. Tsutsui, K. Ukena, M. Takase, C. Kohchi, R.W. Lea, Neurosteroid biosynthesis in vertebrate brains (review), Comp. Biochem. Physiol. C 124 (1999) 121–129.
[6] K. Tsutsui, K. Ukena, M. Usui, H. Sakamoto, M. Takase, Novel brain function: biosynthesis and actions of neurosteroids in neurons (review), Neurosci. Res. 36 (2000) 261–273.
[7] N.A. Compagnone, S.H. Mellon, Neurosteroids: biosynthesis and function of these novel neuromodulators (review), Front. Neuroendocrinol. 21 (2000) 1–56.
[8] K. Tsutsui, Biosynthesis and biological actions of neurosteroids in brain neurons (review), Zool. Sci. 18 (2001) 1043–1053.
[9] C. Corpéchot, P. Robel, M. Axelson, J. Sjövall, E.E. Baulieu, Characterization and measurement of dehydroepiandrosterone sulfate in rat brain, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 4704–4707.
[10] C. Corpéchot, M. Synguelakis, S. Talha, M. Axelson, J. Sjövall, R. Vihko, E.E. Baulieu, P. Robel, Pregnenolone and its sulfate ester in rat brain, Brain Res. 270 (1983) 119–125.
[11] P. Robel, E.E. Baulieu, Neuro-steroids, 3β-hydroxy-Δ^5-derivatives in the rodent brain, Neurochem. Int. 7 (1985) 953–958.
[12] A. Lanthier, V.V. Patwardhan, Sex steroids and 5-en-3β-hydroxysteroids in specific regions of the human brain and cranial nerves, J. Steroid Biochem. 25 (1986) 445–449.
[13] P. Robel, C. Corpéchot, C. Clarke, A. Groyer, M. Synguelakis, C. Vourc'h, E.E. Baulieu, Neuro-steroids: 3β-hydroxy-Δ^5-derivatives in the rat brain, in: G. Fink, A.J. Harmar, K.W. McKerns (Eds.), Neuroendocrine Molecular Biology, Plenum Press, New York, 1986, pp. 367–377.
[14] P. Robel, E. Bourreau, C. Corpéchot, D.C. Dang, F. Halberg, C. Clarke, M. Haug, M.L. Schlegel, M. Synguelakis, C. Vourc'h, E.E. Baulieu, Neuro-steroids: 3β-hydroxy-Δ^5-derivatives in rat and monkey brain, J. Steroid Biochem. 27 (1987) 649–655.
[15] D.H. Jo, M.A. Abdallah, J. Young, E.E. Baulieu, P. Robel, Pregnenolone, dehydroepiandrosterone, and their sulfate and fatty acid esters in the rat brain, Steroids 54 (1989) 287–297.
[16] C. Mathur, V.V.K. Prasad, V.S. Raju, M. Welch, S. Lieberman, Steroids and their conjugates in the mammalian brain, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 85–88.
[17] K. Tsutsui, T. Yamazaki, Avian neurosteroids. I. Pregnenolone biosynthesis in the quail brain, Brain Res. 678 (1995) 1–9.
[18] M. Usui, T. Yamazaki, S. Kominami, K. Tsutsui, Avian neurosteroids. II. Localization of a cytochrome P450scc-like substance in the quail brain, Brain Res. 678 (1995) 10–20.
[19] K. Tsutsui, T. Yamazaki, M. Usui, K. Ukena, S. Kominami, P450scc activity in the brain, in: S. Harvey, R.J. Etches (Eds.), Perspectives in Avian Endocrinology, J. Endocrinol. Ltd., Bristol, 1997, pp. 427–436.
[20] K. Tsutsui, M. Usui, T. Yamazaki, K. Ukena, S. Kominami, Neurosteroids in the avian brain, in: S.K. Maitra (Ed.), Frontiers in Environmental and Metabolic Endocrinology, Burdwan press, Burdwan, 1997, pp. 151–159.
[21] K. Ukena, Y. Honda, Y. Inai, C. Kohchi, R.W. Lea, K. Tsutsui, Expression and activity of 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4-isomerase in different regions of the avian brain, Brain Res. 818 (1999) 536–542.
[22] K. Ukena, Y. Honda, R.W. Lea, K. Tsutsui, Developmental changes in progesterone biosynthesis and metabolism in the quail brain, Brain Res. 898 (2001) 190–194.
[23] M. Matsunaga, K. Ukena, K. Tsutsui, Expression and localization of cytochrome P450 17α-hydroxylase/c17,20-lyase in the avian brain, Brain Res. 899 (2001) 112–122.
[24] M. Matsunaga, K. Ukena, K. Tsutsui, Androgen biosynthesis in the quail brain, Brain Res. 948 (2002) 180–185.
[25] K. Tsutsui, B.A. Schlinger, Steroidogenesis in the avian brain, in: A. Dawson, C.M. Chaturvedi (Eds.), Avian Endocrinology, Narosa Publishing House, New Delhi, 2001, pp. 59–77.
[26] M. Takase, K. Ukena, T. Yamazaki, S. Kominami, K. Tsutsui, Pregnenolone, pregnenolone sulfate and cytochrome P450 side-chain cleavage enzyme in the amphibian brain and their seasonal changes, Endocrinology 140 (1999) 1936–1944.
[27] H. Sakamoto, K. Ukena, K. Tsutsui, Activity and localization of 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4-isomerase isomerase in the zebrafish central nervous system, J. Comp. Neurol. 439 (2001) 291–305.
[28] A.G. Mensah-Nyagan, M. Feuilloley, E. Dupont, J.L. Do-Rego, F. Leboulenger, G. Pelletier, H. Vaudry, Immunocytochemical localization and biological activity of 3β-hydroxysteroid dehydrogenase in the central nervous system of the frog, J. Neurosci. 14 (1994) 7306–7318.
[29] A.G. Mensah-Nyagan, M. Feuilloley, J.L. Do-Rego, A. Marcual, C. Lange, M.C. Tonon, G. Pelletier, H. Vaudry, Localization of 17β-hydroxysteroid dehydrogenase and characterization of testosterone in the brain of the male frog, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 1423–1428.

[30] A.G. Mensah-Nyagan, J.L. Do-Rego, M. Feuilloley, A. Marcual, C. Lange, G. Pelletier, H. Vaudry, In vivo and in vitro evidence for the biosynthesis of testosterone in the telencephalon of the female frog, J. Neurochem. 67 (1996) 413–422.
[31] A. Vanson, A.P. Arnold, B.A. Schlinger, 3β-Hydroxysteroid dehydrogenase/isomerase and aromatase activity in primary cultures of developing zebra finch telencephalon: dehydroepiandrosterone as substrate for synthesis of androstenedione and estrogens, Gen. Comp. Endocrinol. 102 (1996) 342–350.
[32] B.A. Schlinger, N.I. Lane, W. Grisham, L. Thompson, Androgen synthesis in a songbird: a study of cyp17 (17α-hydroxylase/c17,20-lyase) activity in the zebra finch, Gen. Comp. Endocrinol. 113 (1999) 46–58.
[33] F. Freking, T. Nazairians, B.A. Schlinger, The expression of the sex steroid-synthesizing enzymes CYP11A1, 3β-HSD, CYP17, and CYP 19 in gonads and adrenals of adult and developing zebra finches, Gen. Comp. Endocrinol. 119 (2000) 140–151.
[34] C. Le Goascogne, P. Robel, M. Gouézou, N. Sananès, E.E. Baulieu, M. Waterman, Neurosteroids: cytochrome P-450scc in rat brain, Science 237 (1987) 1212–1215.
[35] Z.Y. Hu, E. Bourreau, I. Jung-Testas, P. Robel, E.E. Baulieu, Neurosteroids: oligodendrocyte mitochondria convert cholesterol to pregnenolone, Proc. Natl. Acad. Sci. U.S.A. 84 (1987) 8215–8219.
[36] I. Jung-Testas, Z.Y. Hu, E.E. Baulieu, P. Robel, Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells, Endocrinology 125 (1989) 2083–2091.
[37] V. Papadopoulos, P. Guarneri, K.E. Krueger, A. Guidotti, E. Costa, Pregnenolone biosynthesis in C6-2B glioma cell mitochondria: regulation by a mitochondrial diazepam binding inhibitor receptor, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 5113–5117.
[38] E.E. Baulieu, P. Robel, Neurosteroids: a new brain function? J. Steroid Biochem. Mol. Biol. 37 (1990) 395–403.
[39] E.E. Baulieu, Neurosteroids: a new function in the brain, Biol. Cell 71 (1991) 3–10.
[40] Y. Akwa, J. Young, K. Kabbadj, M.J. Sancho, D. Zucman, C. Vourc'h, I. Jung-Testas, Z.Y. Hu, C. Le Goascogne, D.H. Jo, C. Corpéchot, P. Simon, E.E. Baulieu, P. Robel, Neurosteroids: biosynthesis, metabolism and function of pregnenolone and dehydroepiandrosterone in the brain, J. Steroid Biochem. Mol. Biol. 40 (1991) 71–81.
[41] R.W. Lea, J.A. Clark, K. Tsutsui, Changes in central steroid receptor expression, steroid synthesis and dopaminergic activity related to the reproductive cycle of the ring dove (review), Microsc. Res. Tech. 55 (2001) 12–26.
[42] K. Ukena, M. Usui, C. Kohchi, K. Tsutsui, Cytochrome P450 side-chain cleavage enzyme in the cerebellar Purkinje neuron and its neonatal change in rats, Endocrinology 139 (1998) 137–147.
[43] K. Ukena, C. Kohchi, K. Tsutsui, Expression and activity of 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4-isomerase in the rat Purkinje neuron during neonatal life, Endocrinology 140 (1999) 805–813.
[44] R.B. Oonk, J.S. Krasnow, W.G. Beattie, J.S. Richards, Cyclic AMP-dependent and independent regulation of cholesterol side chain cleavage cytochrome P-450 (P-450scc) in rat ovarian granulosa cells and corpora lutea. cDNA and deduced amino acid sequence of P-450scc, J. Biol. Chem. 264 (1989) 21934–21942.
[45] U. Nudel, R. Zakut, M. Shani, S. Neuman, Z. Levy, D. Yaffe, The nucleotide sequence of the rat cytoplasmic β-actin gene, Nucleic Acids Res. 11 (1983) 1759–1771.
[46] H.F. Zhao, C. Labrie, J. Simard, Y. de Launoit, C. Trudel, C. Martel, E. Rhéaume, E. Dupont, V. Luu-The, G. Pelletier, F. Labrie, Characterization of rat 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4 isomerase cDNAs and differential tissue-specific expression of the corresponding mRNAs in steroidogenic and peripheral tissues, J. Biol. Chem. 266 (1991) 583–593.
[47] H.F. Zhao, E. Rhéaume, C. Trudel, J. Couet, F. Labrie, J. Simard, Structure and sexual dimorphic expression of a liver-specific rat 3β-hydroxysteroid dehydrogenase/isomerase, Endocrinology 127 (1990) 3237–3239.
[48] J. Simard, J. Couet, F. Durocher, Y. Labrie, R. Sanchez, N. Breton, C. Turgeon, F. Labrie, Structure and tissue-specific expression of a novel member of the rat 3β-hydroxysteroid dehydrogenase/Δ^5–Δ^4 isomerase (3β-HSD) family. The exclusive 3β-HSD gene expression in the skin, J. Biol. Chem. 268 (1993) 19659–19668.
[49] H. Sakamoto, K. Ukena, K. Tsutsui, Effects of progesterone synthesized de novo in the developing Purkinje cell on its dendritic growth and synaptogenesis, J. Neurosci. 21 (2001) 6221–6232.
[50] H. Sakamoto, K. Ukena, K. Tsutsui, Dendritic spine formation in response to progesterone synthesized de novo in the developing Purkinje cell in rats, Neurosci. Lett. 322 (2002) 111–115.
[51] A.M. Traish, H.H. Wotiz, Monoclonal and polyclonal antibodies to human progesterone receptor peptide-(533–547) recognize a specific site in unactivated (8S) and activated (4S) progesterone receptor and distinguish between intact and proteolyzed receptors, Endocrinology 127 (1990) 1167–1175.
[52] C.K. Wagner, A.Y. Nakayama, G.J. de Vries, Potential role of maternal progesterone in the sexual differentiation of the brain, Endocrinology 139 (1998) 3658–3661.
[53] S.A. Haywood, S.X. Simonian, E. M van der Beek, R.J. Bicknell, A.E. Herbison, Fluctuating estrogen and progesterone receptor expression in brainstem norepinephrine neurons through the rat estrous cycle, Endocrinology 140 (1999) 3255–3263.
[54] Y. Kastrup, M. Hallbeck, Å. Amandusson, S. Hirata, O. Hermanson, A. Blomqvist, Progesterone receptor expression in the brainstem of the female rat, Neurosci. Lett. 275 (1999) 85–88.
[55] A. Furukawa, A. Miyatake, T. Ohnishi, Y. Ichikawa, Steroidogenic acute regulatory protein (StAR) transcripts constitutively expressed in the adult rat central nervous system: colocalization of StAR, cytochrome P-450scc (CYP XIA1), and 3β-hydroxysteroid dehydrogenase in the rat brain, J. Neurochem. 71 (1998) 2231–2238.
[56] B.J. Clark, J. Wells, S.R. King, D.M. Stocco, The purification cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR), J. Biol. Chem. 269 (1994) 28314–28322.
[57] D.M. Stocco, B.J. Clark, Regulation of the acute production of steroids in steroidogenic cells, Endocrinol. Rev. 17 (1996) 221–244.
[58] J.I. Mason, The 3β-hydroxysteroid dehydrogenase gene family of enzymes, Trends Endocrinol. Metab. 4 (1993) 199–203.
[59] K. Tsutsui, K. Ukena, Neuronal neurosteroidogenesis in the cerebellum, in: M. Okamoto, Y. Ishimura, H. Nawata (Eds.), Molecular Steroidogenesis, Universal Academy Press, Tokyo, 2000, pp. 397–400.
[60] K. Tsutsui, K. Ukena, H. Sakamoto, Novel cerebellar function: neurosteroids in the Purkinje neurons and their genomic and nongenomic actions, in: R.J. Handa, S. Hayashi, E. Terasawa, M. Kawata (Eds.), Neuroplasticity, Development, and Steroid Hormone Action, CRC Press, Boca Raton, 2001, pp. 101–116.
[61] K. Tsutsui, K. Ukena, Neurosteroids in the cerebellar Purkinje neuron and their actions (review), Int. J. Mol. Med. 4 (1999) 49–56.
[62] K. Tsutsui, H. Sakamoto, K. Ukena, A novel aspect of the cerebellum: biosynthesis of neurosteroids in the Purkinje cell (review), Cerebellum, in press.
[63] J. Altman, Postnatal development of the cerebellar cortex in the rat. I. The external germinal layer and the transitional molecular layer, J. Comp. Neurol. 145 (1972) 353–397.
[64] J. Altman, Postnatal development of the cerebellar cortex in the rat. II. Phases in the maturation of Purkinje cells and of the molecular layer, J. Comp. Neurol. 145 (1972) 399–463.