The Journal of Neuroscience, August 15, 2001, 21(16):6221–6232

Effects of Progesterone Synthesized *De Novo* in the Developing Purkinje Cell on Its Dendritic Growth and Synaptogenesis

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De novo steroidogenesis from cholesterol is a conserved property of vertebrate brains, and such steroids synthesized *de novo* in the brain are called neurosteroids. The identification of neurosteroidogenic cells is essential to the understanding of the physiological role of neurosteroids in the brain. We have demonstrated recently that neuronal neurosteroidogenesis occurs in the brain and indicated that the Purkinje cell actively synthesizes several neurosteroids *de novo* from cholesterol in vertebrates. Interestingly, in the rat, this neuron actively synthesizes progesterone *de novo* from cholesterol only during neonatal life, when cerebellar cortical formation occurs most markedly. Therefore, in this study, the possible organizing actions of progesterone during cerebellar development have been examined. *In vitro* studies using cerebellar slice cultures from newborn rats showed that progesterone promotes dose-dependent dendritic outgrowth of Purkinje cells but dose not affect their somata. This effect was blocked by the anti-progestin RU 486 [mifepristone; 17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl) estra-4,9-dien-3 one-6–7]. *In vivo* administration of progesterone to pups further revealed an increase in the density of Purkinje spine synapses electron microscopically. In contrast to progesterone, there was no significant effect of 3α,5α-tetrahydroprogesterone, a progesterone metabolite, on Purkinje cell development. Reverse transcription-PCR–Southern and immunocytochemical analyses showed that intranuclear progesterone receptors were expressed in Purkinje cells. These results suggest that progesterone promotes both dendritic outgrowth and synaptogenesis in Purkinje cells through intranuclear receptor-mediated mechanisms during cerebellar development. Such organizing actions may contribute to the formation of the cerebellar neuronal circuit.

Key words: Purkinje cell; neurosteroids; progesterone; 3α,5α-tetrahydroprogesterone; progesterone receptor; genomic action; dendritic outgrowth; synaptogenesis; cerebellar cortical formation; development

Peripheral steroid hormones act on brain tissues through intracellular receptor-mediated mechanisms to regulate several important brain neuronal functions (Fuxe et al., 1981; Arnold and Gorski, 1984). Therefore, the brain is considered to be a target site of steroid hormones. However, it is now established in a number of vertebrate species that the brain itself also synthesizes steroids *de novo* from cholesterol through mechanisms at least partly independent of peripheral steroidogenic glands (Baulieu, 1997; Tsutsui et al., 1999, 2000). Such steroids synthesized *de novo* in the brain, as well as other areas of the nervous system, are called neurosteroids (Corpéchot et al., 1981; Le Goascogne et al., 1987). The identification of neurosteroidogenic cells is essential to the understanding of the physiological role of neurosteroids in brain function. Glial cells are generally accepted to be the major site for neurosteroid formation (Hu et al., 1987; Akwa et al., 1991; Baulieu, 1991), but the concept of neurosteroidogenesis in brain neurons has, up to now, been uncertain. The cytochrome P450 side-chain cleavage enzyme (P450scc) cleaves cholesterol to form pregnenolone, and 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) catalyzes the dehydrogenation and isomerization of pregnenolone into progesterone. Recently, our studies have indicated that Purkinje cells possess these steroidogenic enzymes in a variety of vertebrates (for review, see Tsutsui et al., 2000). Cytochrome P450scc appears in the rat Purkinje cell immediately after differentiation and the expression of this enzyme persists during neonatal development into adulthood, suggesting a constant production of pregnenolone (Ukena et al., 1998). In contrast, the expression of 3β-HSD increases during the neonatal period, unlike P450scc (Ukena et al., 1999a). Such an age-dependent expression of 3β-HSD has been confirmed by biochemical studies, indicating an increase of progesterone formation during neonatal life (Ukena et al., 1999a). Notwithstanding such a difference in age-dependent expression, our studies have demonstrated that the Purkinje cell, a typical cerebellar neuron, is an important neurosteroidogenic cell. To our knowledge, this is the first observation of neuronal neurosteroidogenesis in the brain.

It is well known that dramatic morphological changes in the rat cerebellum occur after birth during neonatal life (Altman, 1972a,b). Rat Purkinje cells differentiate just after birth, and the formation of the cerebellar cortex becomes complete in the neonate, through the 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 cerebellar progesterone is high (Ukena et al., 1999a).

Received Jan. 17, 2001; revised April 23, 2001; accepted May 18, 2001.

This work was supported in part by the Ministry of Education, Science, Sports, and Culture of Japan (Grants-in-Aid for Scientific Research 11170237, 11354010, 12440233, 12894021, and 13210101 to K.T.). H.S. is supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists. We are grateful to Dr. Robert W. Lea (University of Central Lancashire, Preston, UK) for his valuable discussion and for reading this manuscript.

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More recently, we have also identified some metabolite(s) of progesterone, such as 3α,5α-tetrahydroprogesterone (THP), in the cerebellum of neonatal rats (Tsutsui et al., 2000). Accordingly, progesterone and/or its metabolite(s) may be involved in the formation of the cerebellar neuronal circuit that occurs during neonatal life through promoting neuronal growth and neuronal synaptic contact by genomic actions.

To test this hypothesis, we examined the effect of progesterone on neuronal growth and synaptogenesis in the rat cerebellum. To reveal the mode of progesterone action, we further investigated the expression and localization of progesterone receptors (PR) and the action of 3α,5α-THP, a progesterone metabolite.

MATERIALS AND METHODS

Animals. Male and female rats of the Fisher strain maintained in this laboratory were mated and housed in a temperature-controlled room (25 ± 2°C) under a daily photoperiod of 14/10 hr light/dark cycle (lights on at 6:00 A.M.). Newborn male rats of 3 and 5 d of age, when endogenous progesterone and its metabolite (3α,5α-THP) were very low in the cerebellum (Ukena et al., 1999a; Tsutsui and Ukena, 2000; Tsutsui et al., 2000), were prepared as subjects for *in vitro* and *in vivo* treatments with neurosteroids. In previous studies (Ukena et al., 1999a; Tsutsui et al., 2000), the expression of 3β-HSD in Purkinje cells was negligible at 3 d of age but increased from 7 d to reach a peak at ~10 d and decreased thereafter. Such changes were consistent with changes in cerebellar progesterone (Ukena et al., 1999a; Tsutsui et al., 2000). Postnatal rats of various ages and both sexes were also used to study the expression of nuclear PR. The experimental protocol was approved in accordance with the Guide for the Care and Use of Laboratory Animals prepared by Hiroshima University (Higashi-Hiroshima, Japan).

Slice culture of cerebella. Cerebella of male pups at 5 d of age were used for organotypic slice cultures. After decapitation under deep anesthesia, cerebella were dissected out into ice-cold HBSS, pH 7.3, and embedded in 2% low-gelling-temperature (30–31°C) agar in HBSS at 35–38°C. Immediately after embedding, the preparation was cooled to 4°C to facilitate the gelling of the agar in <1 min. Vermal parasagittal slices (400 μm thick) were cut on a microslicer. Cultures of cerebellar slices were conducted according to a method of hippocampal organotypic cultures by Stoppini et al. (1991). In brief, cerebellar slice tissues were cultured on a porous membrane (Intercell TP; Kurabo), which was floated at the interface between air and a culture medium in a 24-hole well. The culture medium was a modification of medium described previously (Bottenstein and Sato, 1979; Messer et al., 1981; Gruol and Crimi, 1988; Tanaka et al., 1994) and composed of a 1:1 mixture of DMEM and Ham's F-12 (Sigma, St. Louis, MO), 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) for the first 2 d of culture [2 d *in vitro* (DIV)]. Cultures were maintained at 37°C in an atmosphere of humidified 95% air and 5% CO_2.

In vitro *steroid treatment.* To investigate morphological changes of Purkinje cells induced by progesterone or 3α,5α-THP, these neurosteroids were applied to granuloprival cerebellar cultures for 3 d after 2 DIV, and cultures were fixed at 5 DIV. Crystalline progesterone or 3α,5α-THP (Sigma) was dissolved into absolute ethanol and applied to the culture medium at various concentrations (progesterone, 0.1, 1, 10, and 100 nM and 1 μM; 3α,5α-THP, 1, 10, and 100 nM). The final concentration of ethanol was adjusted to 0.001% (v/v) in all steroid-treated and control (vehicle alone) groups. The effect of an antiprogestin, RU 486 [mifepristone; 17β-hydroxy-11β-(4-methylaminophenyl)-17α-(1-propynyl) estra-4,9-dien-3 one-6–7] (Biomol, Plymouth Meeting, PA), 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 (PFA), 2.5% glutaraldehyde (GA), and 15% saturated picric acid (v/v) in 0.1 M phosphate buffer (PB), pH 7.3, overnight at 4°C and subjected to immunocytochemical labeling of Purkinje cells using a calcium-binding protein (calbindin) antibody as described below, followed by the morphological analysis of Purkinje cells.

In vivo *steroid treatment.* Progesterone dissolved in sesame oil (50 μg/25 μl) was injected into the reticulospinal fluid around the posterior vermis of male pups once per day for 4 d from 3 d of age. Pups receiving injections of the vehicle alone (sesame oil) served as controls. At 7 d of age, pups were deeply anesthetized with chloroform before transcardial perfusion with PBS (10 mM PB and 0.14 M NaCl, pH 7.3), followed by fixative solution [2% PFA, 2.5% GA, 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.

To investigate the effect of endogenous progesterone on dendritic development, RU 486 was injected to male pups during 7–10 d of age, when the endogenous progesterone level was maximal (Ukena et al., 1999). RU 486 (2 mg) dissolved in dimethylsulfoxide (DMSO) (5 μl) was added to 995 μl of sesame oil (final concentration is 50 μg/25 μl) and injected into the reticulospinal fluid around the posterior vermis of male pups once per day for 4 d from 7 d of age. Pups receiving injections of the vehicle alone (0.5% DMSO in sesame oil) served as controls. Pups at 11 d of age were also used for morphological analyses.

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), shown previously to label Purkinje cells specifically in organotypic cultures (Schilling et al., 1991; Tauer et al., 1996, 1998) and *in vivo* studies (Stottmann and Rivas, 1998). Cerebellar sections and slice cultures were prepared as described above and 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, Burlingame, CA), followed by diaminobenzidine (DAB) reaction with a slight modification of the instructions of the manufacturer, as previously described (Ukena et al., 1998; Takase et al., 1999; Sakamoto et al., 2000). After dehydration, stained sections and slice cultures were studied using an Olympus Optical (Tokyo, Japan) BH-2 microscope.

Light microscopic analysis of the morphology of Purkinje cells. After immunostaining for calbindin, 5–10 labeled Purkinje cells with dendrites and axons visible were randomly selected in each vermal lobe of slice cultures derived from *in vitro* studies. Five to 10 labeled Purkinje cells were also randomly selected in the vermal lobe IX around the site of *in vivo* steroid injection. The selected Purkinje cell was traced (magnification, 800×) 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 cross-sectional soma area of Purkinje cells, the border between somatic and proximal dendritic membrane was defined as the point at which the spherical shape of the somatic membrane was broken by the origin of a proximal dendrite. To measure the dendritic area of Purkinje cells, cross-sectional soma area was deducted from whole area of each cell. Differences in the morphological appearance of Purkinje cells after treatment with progesterone or 3α,5α-THP were analyzed by a Student's *t* test between two different groups or a one-way ANOVA among more than two different groups (Bliss, 1952). If significance was reached with the ANOVA test, the analysis was followed by a Duncan's multiple range test (Bliss, 1952). All *in vitro* treatment groups were composed of the cultured slices from multiple animals (at least four animals), and all slices were separately cultured in the individual chamber. Statistical comparisons of *in vitro* studies (see Figs. 2–4, Table 1) were based on the individual slice as the unit of analysis, because the physiological environment in tissue culture rapidly becomes independent of the animal of origin. Statistical analyses of *in vivo* studies (see Figs. 5–7, Table 2) were based on the individual animal.

To analyze the effects of progesterone (10^{-8} M) 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) (magnification, 800×), representing the exact length and complexity of the dendritic arbor (Shimada et al., 1998). Five to 10 labeled 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 and expressed as mean ± SEM. To be selected for analysis of dendritic spine density, dendritic segments had to meet several criteria: they had to

Table 1. Effects of progesterone on the number of dendritic spines and total dendritic length of Purkinje cells

Treatment	Number of slices	Number of rats	Number of dendritic spines (spines/50 μm dendrite)	Total dendritic length (μm/cell)
Vehicle	12	4	11.6 ± 0.9	116.6 ± 10.5
Progesterone	12	4	14.6 ± 0.9*	188.2 ± 18.2**

*$p < 0.05$ and **$p < 0.01$ compared with vehicle (by Student's t test).

Table 2. Comparison of progesterone levels in the cerebellum between vehicle- and progesterone-treated groups

Treatment	Number of rats	Cerebellar weight (mg)	Progesterone concentration (fmol/mg)
Vehicle	4	29.6 ± 0.6	15.3 ± 5.7
Progesterone	5	29.8 ± 1.7	244.1 ± 62.9*

*$p < 0.05$ compared with vehicle (by Student's t test).

be easily identifiable as belonging to the Purkinje cell that was both thoroughly immunostained with calbindin and clearly distinguishable from neighboring immunoreactive cells, be located in the cerebellar lobe IX, remain approximately in one plane of focus, and be >15 μm in length. For each dendritic segment selected, spine density was measured as follows: the selected segment was traced (magnification, 1200×) with a camera lucida drawing tube; all of 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 50 μm dendrite. Three to five dendritic segments per cell and at least six Purkinje cells per slice were analyzed. Differences in the dendritic spine number and total dendritic length of Purkinje cells after treatment with vehicle or progesterone were analyzed by a Student's t test (Bliss, 1952).

To verify the action of endogenous progesterone on Purkinje dendritic outgrowth, pups injected with the anti-progestin RU 486 during the endogenous peak of progesterone (7–10 d of age) were used for morphological analysis. The length of molecular layer was evaluated as a parameter of maximal dendritic length (see Fig. 6*A*). At least 16 regions from four calbindin-immunostained sections per animal were randomly selected in the vermal lobe IX around the site of *in vivo* injection. The maximal dendritic length was measured using an ocular micrometer (magnification, 800×) under an Olympus Optical BH-2 microscope. Differences in the maximal dendritic length of Purkinje cells after *in vivo* treatment with vehicle or RU 486 were analyzed by a Student's t test (Bliss, 1952).

Electron microscopic analysis of the morphology of Purkinje cells. For electron microscopy, calbindin immunocytochemically stained lobe sections of progesterone- ($n = 6$) and vehicle- ($n = 6$) treated male pups at 7 d of age were post-fixed in 1% osmium tetroxide in PB, dehydrated in ascending grades of ethanol, and then embedded flat in epoxy resin (Quetol-812; Nisshin EM) according to our previous method (Sakamoto et al., 2000). 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). All electron microphotographs were coded and evaluated without knowledge of the experimental group, and the code was not broken until the analysis was complete. At least 24 electron microphotographs (photographed at 6000× and printed at 15,000× magnification) of random regions in the molecular layer of lobe IX were generated from six different animals per each experimental group. The number of asymmetrical synapses, defined as having both a postsynaptic density and at least three synaptic vesicles in the presynaptic terminal no >0.2 μm from the synaptic cleft, was counted. The asymmetrical synapses on Purkinje dendritic spines were counted separately from those on dendritic shafts. Because relatively few cross-sections contained a spine neck or spine apparatus, spines were defined as postsynaptic processes smaller than 2 μm in diameter, lacking mitochondria (Woolley and McEwen, 1992; Bravin et al., 1999). The length of each synaptic membrane was measured using an NIH Image software package from electron micrographs. The density of synapses per volume (*estNsv*) was calculated using an unfolding stereological method of Cruz-Orive (1983), according to the formula $estNsv = Qs/(Ds \cdot \pi/4 + t)$, where *estNsv* is the number of synapses per unit volume, *Qs* is the number of synapses per unit area, *Ds* is the mean length of synaptic contact zones, and t is the thickness of the ultrathin section (average thickness, 60 nm) (Cruz-Orive, 1983). Statistical differences for the number of synapses per unit volume between steroid- and vehicle-treated groups were analyzed by a Student's t test (Bliss, 1952).

Radioimmunoassay of progesterone. Progesterone levels in the cerebellum of steroid- ($n = 5$) or vehicle- ($n = 4$) treated male pups were measured at 7 d of age, when morphological changes in the Purkinje cells were analyzed *in vivo*. Male pups were killed between 10:00 and 11:00 A.M., and cerebella were frozen in liquid nitrogen and stored at −80°C. Extraction of progesterone was performed according to a method described previously (Tsutsui and Yamazaki, 1995; Tsutsui et al., 1998; Ukena et al., 1998, 1999a,b). Briefly, cerebella were homogenized in 5 ml of ice-cold PBS with a Teflon-glass homogenizer and then subjected to steroid extraction. To estimate steroid recovery during extraction, 2000 cpm of [1,2,6,7-^{3}H]progesterone (specific activity, 115 Ci/mmol; NEN, Boston, MA) was added to each sample with 5 ml of ethyl acetate. The tubes were stirred for 30 min and centrifuged at 3000 × g for 5 min, and the organic phase was removed. This extraction step was repeated twice. The combined organic extracts, which contained progesterone, were dried down and dissolved in 1 ml of PBS containing 0.1% gelatin. The aqueous solution was divided into two aliquots: one aliquot for the estimation of recovery, the other for the measurement of progesterone. To measure progesterone concentration, aliquots of the organic extracts were assayed in a progesterone radioimmunoassay (RIA) (Corpéchot et al., 1983; Nudel et al., 1983; Tsutsui and Yamazaki, 1995; Tsutsui et al., 1998; Ukena et al., 1998, 1999a) using an antiserum to progesterone (Scantibodies Laboratory Inc., Santee, CA) and [1,2,6,7-^{3}H]progesterone. The antiserum used in this assay cross-reacted with deoxycorticosterone at 3.3%, 17α-hydroxyprogesterone at 0.6%, and pregnenolone at <0.1%, and no chromatographic purification of progesterone was performed. Separation of bound and free steroid was performed by centrifugation after reaction with the IgG SORB (The Enzyme Center Inc.). The least detectable amount of progesterone was 0.1 ng/ml, and intra-assay variation was estimated as <7%. The precision index (λ) of a linear portion of the competition curve, computed according to a method described previously (Tsutsui, 1991; Tsutsui and Yamazaki, 1995), was 0.037. Results of the RIA were expressed as mean ± SEM. Statistical comparisons of progesterone concentrations between progesterone- and vehicle-treated groups were made by a Student's t test.

Reverse transcription-PCR analysis of PR mRNA. To determine the expression of mRNA encoding for rat PR in the cerebellum, reverse transcription (RT)-PCR analysis was performed using rat tissue collected during neonatal development and from adults according to our previous method (Ukena et al., 1998, 1999a,b). In this study, rats at 0, 3, 7, 14, 21, and 60 d of age ($n = 4$ at each age, of both sexes) were killed between 10:00 and 11:00 A.M. Total RNA (including ribosomal RNA and mRNA) from the cerebellum of each rat was isolated by the guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). The average amount of total RNA extracted from one cerebellum was 71 μg on day 0, 93 μg on day 3, 101 μg on day 7, 342 μg on day 14, 318 μg on day 21, and 235 μg on day 60. Thirty micrograms of total RNA were reverse transcribed using oligo-dT primer and RT in a 60 μl reaction volume for 1.5 hr at 37°C. The reaction mixture was composed of 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 mix, 1.5 μg of oligo-dT (Amersham Pharmacia Biotech, Uppsala, Sweden), 15 U of ribonuclease inhibitor (Wako Chemicals, Osaka, Japan), and 400 U of Moloney murine leukemia virus transcriptase (Life Technologies, Gaithersburg, MD). After the reaction was stopped by incubation at 67°C for 10 min, the cDNA was ethanol precipitated and redis-

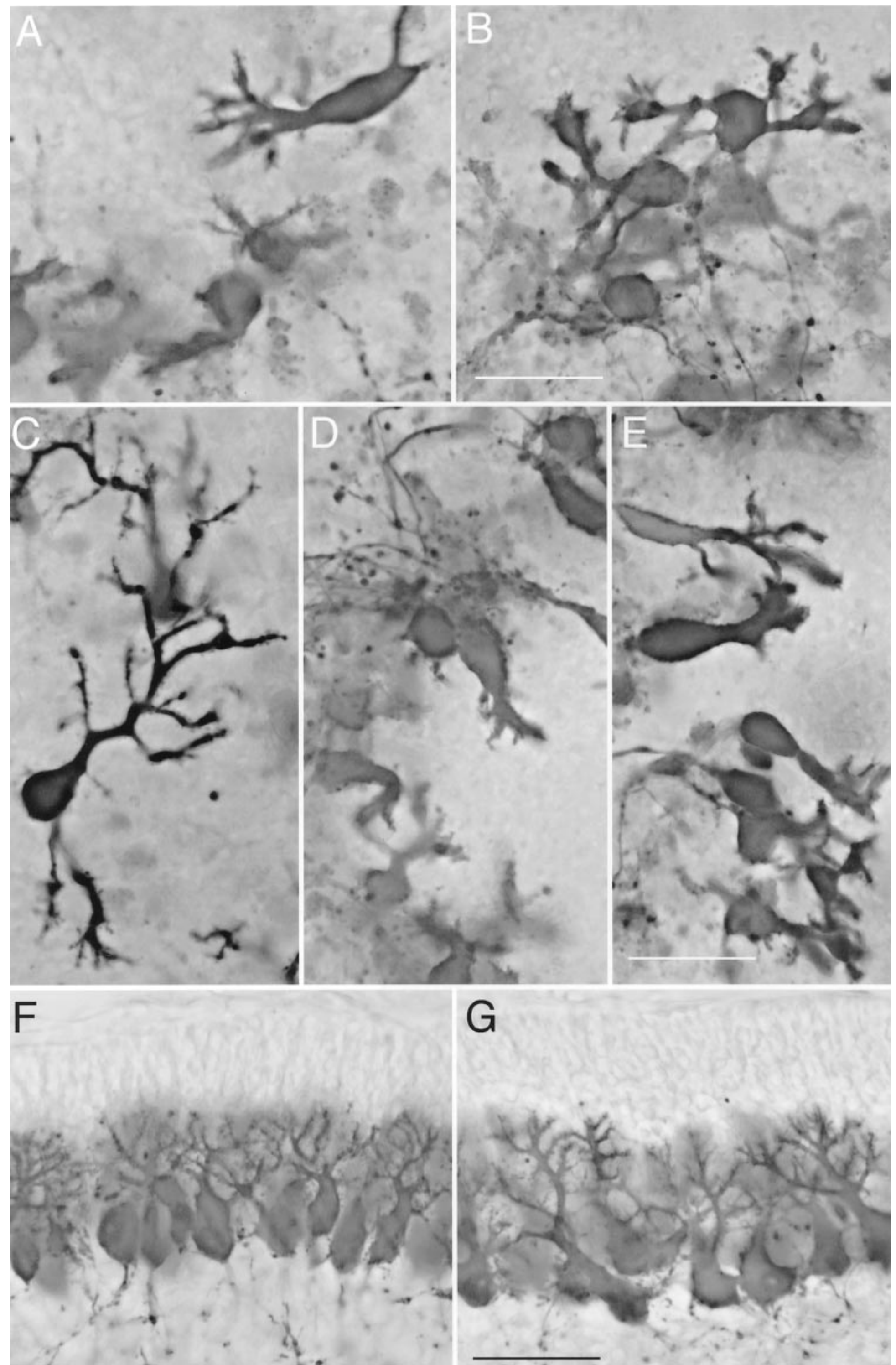

Figure 1. Morphology of Purkinje cells and its modulation by progesterone treatment: *in vitro* and *in vivo* study. *A–E*, Cerebellar cultures from newborn male rats grown for 5 DIV and immunostained for calbindin. Cultures treated with vehicle (*A*), 100 nM progesterone (*B*), 10 nM progesterone (*C*), 1 μM RU 486 alone *(D)*, and 10 nM progesterone plus 1 μM RU 486 (*E*) for 3 d. Progesterone promoted dendritic outgrowth of Purkinje cells *in vitro*, and the effect was blocked by the antiprogestin RU 486. *F*, *G*, Parasagittal sections of neonatal cerebellum at 7 d of age were immunostained for calbindin. Male pups received daily injections of the vehicle (*F*) or progesterone (*G*) for 4 d. Purkinje cells in the progesterone group (*G*) demonstrated more differentiated dendrites compared with the control group (*F*) *in vivo*. All photomicrographs are of the same magnification. Scale bars, 50 μm.

solved in 30 μl of distilled water. For PCR, an aliquot of the cDNA solution corresponding to 0.1 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 deoxynucleoside triphosphate mix, 0.5 μM of each primer, and 1 U of recombinant *Taq* DNA polymerase (Toyobo, Tokyo, Japan). After denaturation at 95°C for 3 min, the mixture was subjected to 30 thermal cycles in a programmed temperature control system (PC700; Astec, Fukuoka, Japan) as follows: denaturation at 93°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. After thermal cycling, the mixture was additionally incubated at 72°C for 10 min. An 8 μl aliquot of each sample was electrophoresed through a 1.5% agarose gel. To confirm the identity of the amplified fragment, the gels were subjected 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 Mannheim, Mannheim, Germany). Oligonucleotides used as PCR primer and probe for mRNA detection, based on nucleotide sequences of rat PR (Park and Mayo, 1991; Park-Sarge and Mayo, 1994) and rat β-actin (Nudel et al., 1983), were as follows: PR sense primer, 5′-CCCACAGGAGTTTGTCAAGCTC-3′; PR antisense primer, 5′-TAACTTCAGACATCATT-

TCCGG-3′; PR probe, 5′-GTTCACAACGCTTCTATCAA-3′; β-actin sense primer, 5′-GAGACCTTCAACACCCCAGC-3′; and β-actin antisense primer, 5′-CACAGAGTACTTGCGCTCAG-3′.

It has been reported previously that rat PR has two different isoforms (type A and B) (Kato et al., 1993, 1994; Camacho-Arroyo et al., 1998; Guerra-Araiza et al., 2000; Szabo et al., 2000). In this study, the rat PR sense and antisense primers, which are identical and complementary to a common sequence of type A and type B (ligand-binding domain), give a 326 bp amplified fragment of the PR gene. The β-actin primers give a 645 bp amplified fragment located in exons 3–6.

Immunocytochemistry of PR. In this immunocytochemical experiment, neonatal males at 7 d of age ($n = 5$) and adult males at 60 d of age ($n = 5$) were deeply anesthetized with chloroform before transcardial perfusion with PBS, followed by fixative solution (4% PFA in PB). Vermal cerebella were removed, stored in fixative overnight (3.75% acrolein and 2% PFA 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. Every third section was grouped in a single batch of ice-cold PBS so as to obtain three independent series of equally spaced sections. Only one of these series of sections was used for immunocytochemical staining with PR, whereas the remaining two series were used as control staining for the immunocytochemistry and for Nissl-staining, respectively. 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 hr. They were then immersed for 72 hr at 4°C with a polyclonal rabbit antiserum (1:1000) directed against the DNA-binding domain of the human PR (amino acids 533–547, GLPQVYPPYLNYLRP; Dako, High Wycombe, UK). The antiserum used in this experiment cross-reacts with both isoforms (type A and B), and its specificity has been described previously (Traish and Wotiz, 1990; Wagner et al., 1998; Haywood et al., 1999; Kastrup et al., 1999). Immunoreactive products were detected with an ABC kit (Vectastain Elite kit; Vector Laboratories), followed by DAB reaction with a slight modification of the instructions of the manufacturer, as described previously (Ukena et al., 1998; Takase et al., 1999; Sakamoto et al., 2000). 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.

RESULTS

In vitro analysis of Purkinje cell development with progesterone or 3α,5α-THP treatment

To investigate whether progesterone or 3α,5α-THP (a progesterone metabolite), produced actively as neurosteroids in the Purkinje cell during neonatal life, is involved in the growth of Purkinje cells, morphological changes of Purkinje cells were measured after treatment with progesterone or 3α,5α-THP using cerebellar slice cultures of newborn male rats. Initially, progesterone (100 nM) was added to cerebellar cultures in serum-free medium for 3 d after a 2 d incubation period with 5% fetal bovine serum to abolish the excessive cell loss in cultured cerebellar slices. After 5 DIV, most Purkinje cells had survived in the serum-free medium with or without progesterone (Fig. 1*A*,*B*). The morphology of the Purkinje cells in the progesterone-treated group ($n = 18$ slices from 6 different males) (Fig. 1*B*) was compared with that in the vehicle-treated group ($n = 18$ slices from 6 different males) (Fig. 1*A*). Morphological analysis revealed that progesterone administration induced significant increases in the perimeter ($p < 0.001$) (Fig. 2*A*) and dendrite area ($p < 0.001$) (Fig. 2*C*) of Purkinje cells. In contrast, the cross-sectional cell body area of Purkinje cells was unchanged after progesterone treatment (Fig. 2*B*).

Progesterone administration increased, in a dose-related way, the perimeter (Fig. 3*A*) and dendrite area (Fig. 3*C*) of Purkinje cells ($n = 12$ slices from 4 different males in each dose) with a threshold concentration ranging between 1 and 10 nM, indicating that progesterone actions were within the physiological range observed previously during normal cerebellar development (Ukena et al., 1999a). In addition, the stimulatory action of progesterone tended to be decreased at high-dose (100 nM and 1 μM) treatment (Fig. 3*A*,*C*). However, progesterone did not influence the cross-sectional Purkinje cell body area after progesterone treatment at any dose (Fig. 3*B*). In contrast to progesterone, 3α,5α-THP, a progesterone metabolite, failed to significantly alter any morphological parameters with similar dose treatments ($n = 12$ slices from 4 different males in each dose) (Fig. 3). To analyze the effect of progesterone (10^{-8} M) on Purkinje dendritic morphology, we further measured the dendritic spine number and total dendritic length of Purkinje cells in cultured slice at the light microscopic level. As shown in Table 1, progesterone administration resulted in significant increases not only in the num-

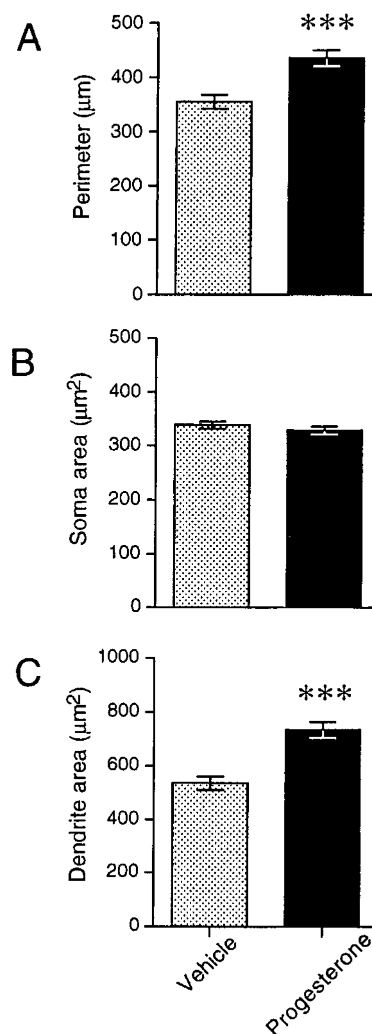

Figure 2. Morphological comparison of Purkinje cells from vehicle- and progesterone-treated groups: *in vitro* study. Perimeter (*A*), soma area (*B*), and dendrite area (*C*) of Purkinje cells were measured after immunostaining for calbindin. Progesterone administration *in vitro* induced significant increases in the perimeter and dendrite area of Purkinje cells but did not affect their somata. Each *column* and error bar represent the mean ± SEM ($n = 18$ slices from 6 different males in each group). Data were derived from 120 Purkinje cells in each group. ***$p < 0.001$ (by Student's *t* test).

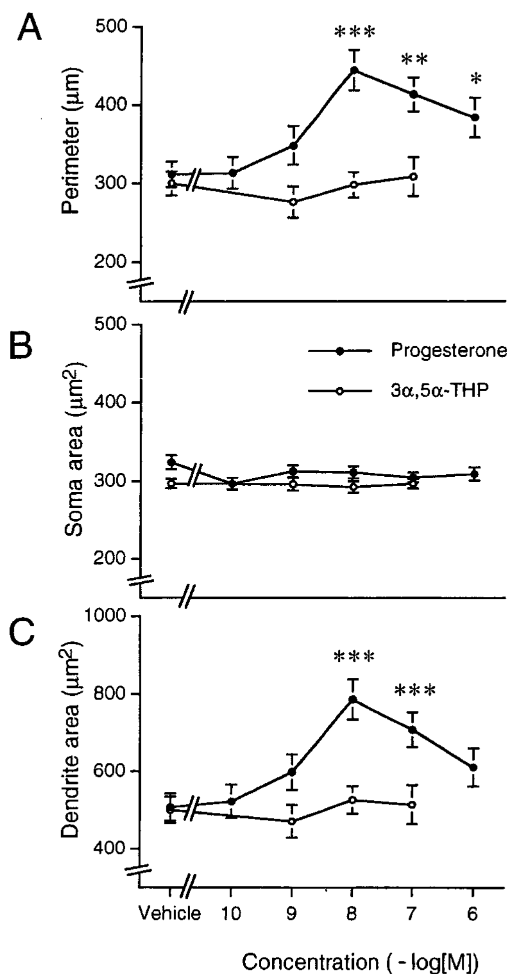

Figure 3. Dose–response of progesterone and its metabolite 3α,5α-THP: *in vitro* study. Perimeter (*A*), soma area (*B*), and dendrite area (*C*) of Purkinje cells were measured after immunostaining for calbindin. Progesterone administration *in vitro* increased, in a dose-related manner, the perimeter and dendrite area of Purkinje cells, unlike their soma area. In contrast to progesterone, 3α,5α-THP, a progesterone metabolite, failed to alter any of the morphological parameters under a similar dose treatment. Each *dot* and error bar represent the mean ± SEM ($n = 12$ slices from 4 different males in each group). Data were derived from 80 Purkinje cells in each group. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus vehicle (by Student's *t* test).

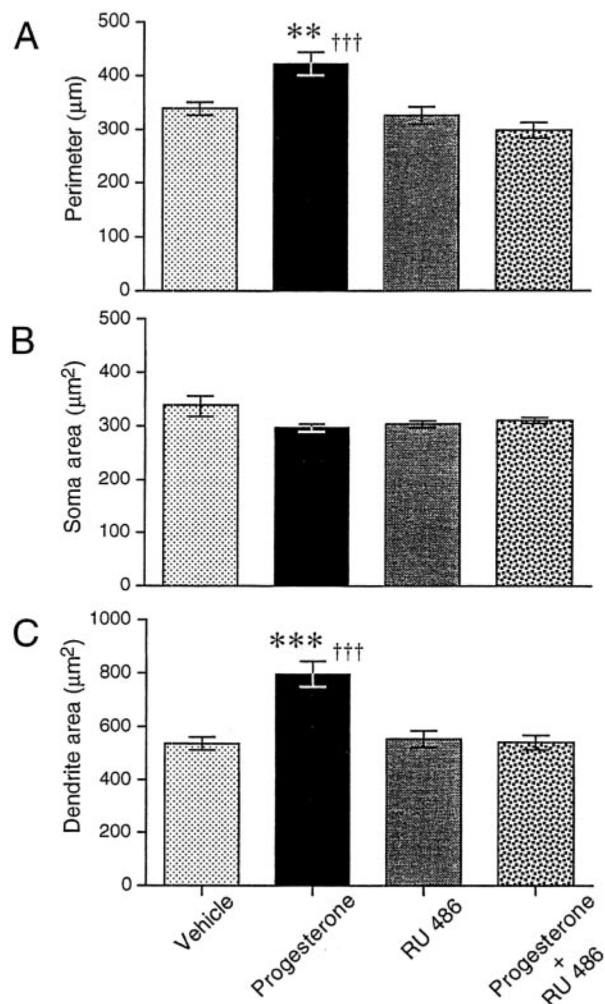

Figure 4. Effect of the anti-progestin RU 486 on Purkinje cell morphology: *in vitro* study. Perimeter (*A*), soma area (*B*), and dendrite area (*C*) of Purkinje cells were measured after immunostaining for calbindin. Treatment with 10 nM progesterone alone *in vitro* caused significant increases in Purkinje perimeter (*A*) and dendrite area (*C*), whereas RU 486 alone at a concentration of 1 μM did not alter any significant differences from the vehicle group. In contrast, combined treatments with progesterone and RU 486 showed that RU 486 abolished the progesterone effect *in vitro*. Each *column* and error bar represent the mean ± SEM ($n = 18$ slices from 6 different males in each group). Data were derived from 120 Purkinje cells in each group. **$p < 0.01$ and ***$p < 0.001$ versus vehicle; †††$p < 0.001$ versus progesterone plus RU 486 group (by one-way ANOVA, followed by Duncan's multiple range test).

ber of dendritic spines per unit length of dendrite (50 μm) ($p < 0.05$) but also in the total length of dendrites ($p < 0.01$) of Purkinje cells ($n = 12$ slices from 4 different males in each treatment).

Subsequently, we investigated whether the effect of progesterone is blocked by an antagonist of PR, RU 486 ($n = 18$ slices from 6 different males in each group) (Fig. 4). Treatment with 10 nM progesterone alone (Fig. 1*C*) induced significant increases in the Purkinje perimeter ($p < 0.01$ vs vehicle group) (Fig. 4*A*) and dendrite area ($p < 0.001$ vs vehicle group) (Fig. 4*C*), whereas RU 486 alone at a concentration of 1 μM (100 times greater than the progesterone concentration) (Fig. 1*D*) did not induce any significant differences compared with the vehicle-treated group (Fig. 4*A,C*). In contrast, combined treatments with progesterone (10 nM) and RU 486 (1 μM) (Fig. 1*E*) revealed that RU 486 abolished the progesterone-induced dendritic outgrowth of Purkinje cells ($p < 0.001$ vs progesterone group) (Fig. 4*A,C*). This would suggest that the progesterone effect was specifically mediated by PR. Unlike morphological changes in Purkinje dendrites, the cross-sectional Purkinje cell body area remained unchanged in this experiment (Fig. 4*B*).

In vivo analysis of Purkinje cell development induced by progesterone administration

Although administration of progesterone promoted the outgrowth of Purkinje cell dendrites *in vitro*, an *in vivo* effect is still unclear. Therefore, this experiment was designed to verify progesterone action on Purkinje dendritic outgrowth *in vivo*. Progesterone was directly injected into the reticulospinal fluid around the posterior vermal lobe (IX) of the cerebellum at 50 μg/d for 4 d during the early neonatal period (from 3 to 6 d of age). This is a period of time when an active formation of endogenous

progesterone does not occur in the Purkinje cell (Ukena et al., 1999a). The morphology of Purkinje cells at 7 d of age was compared between the progesterone- ($n = 6$) and vehicle- ($n = 6$) treated groups after immunostaining for calbindin. Although the lobulation of the vermal cerebellum was maintained in both groups, Purkinje cells in the progesterone-treated group (Fig. 1*G*) possessed more differentiated dendrites compared with the control group (Fig. 1*F*). Morphological analysis further revealed that progesterone administration exhibited significant increases in the perimeter ($p < 0.01$) (Fig. 5*A*) and dendrite area ($p < 0.01$) (Fig. 5*C*) of Purkinje cells, unlike the cross-sectional cell body area (Fig. 5*B*). These data are thus consistent with the results obtained *in vitro*.

In this experiment, progesterone concentrations in the cerebellum were measured in the vehicle- ($n = 4$) and progesterone- ($n = 5$) treated groups using a specific progesterone RIA. Cerebellar progesterone levels in the progesterone-treated group were significantly higher ($p < 0.05$) than those in the control group (Table 2). The level of progesterone after progesterone administration to newborn rats was higher but not significantly different from the maximal physiological level (141.6 ± 47.6 fmol/mg cerebellum) observed previously in neonatal rats at 10 d of age under normal development (Ukena et al., 1999a).

In vivo analysis of Purkinje cell development inhibited by anti-progestin administration

Subsequently, this experiment was designed to verify the action of endogenous progesterone on Purkinje dendritic outgrowth during the endogenous peak of progesterone. RU 486, an antagonist of progesterone receptor, was directly injected into the reticulospinal fluid around the posterior vermal lobe (IX) of the cerebellum at 50 μg/d during 7–10 d of age. This is a period of time when cerebellar progesterone is high (Ukena et al., 1999a). The dendritic morphology of Purkinje cells at 11 d of age was compared between vehicle- ($n = 4$) and RU 486- ($n = 4$) treated groups after immunostaining for calbindin. As shown in Figure 6*A*, administration of the anti-progestin RU 486 tended to inhibit dendritic outgrowth of the Purkinje cell during the endogenous peak of progesterone. Therefore, we evaluated the length of molecular layer as a parameter of maximal dendritic length (Fig. 6*A*), because Purkinje cell dendrites at 11 d of age were well developed and were not clearly distinguishable from neighboring immunoreactive cells. When Purkinje cells were treated with RU 486, the maximal dendritic length decreased significantly ($p < 0.05$) compared with that of vehicle-treated group (Fig. 6*B*).

Progesterone promotes dendritic spine proliferation and synaptic formation of Purkinje cells during development

In vitro and *in vivo* experiments revealed that progesterone treatment promotes the outgrowth of Purkinje cell dendrites, unlike its metabolite 3α,5α-THP. Morphological changes in dendrites of the calbindin-labeled Purkinje cell were further analyzed ultrastructurally using an electron microscope (Fig. 7). Interestingly, qualitative examination at this level showed an increase in the number of Purkinje dendritic spines (Fig. 7*A*). Furthermore, quantitative electron microscopic analysis using an unfolding method revealed that the density of axospinous synapses on Purkinje cells in the progesterone-treated group ($n = 6$) was significantly larger ($p < 0.01$) than that in the vehicle-treated group ($n = 6$) (Fig. 7*C*). Progesterone administration was followed by a 41% increase in the density of synapses on Purkinje dendritic spines compared with the control group (Fig. 7*C*). However, there was no significant difference in the density of synapses located on Purkinje dendritic shafts after treatment (Fig. 7*C*). In addition, no significant differences in other morphological parameters, such as presynaptic dense projection (*pdp*), postsynaptic density (*psd*), and synaptic vesicle (*sv*), were observed between the two groups (Fig. 7*B*).

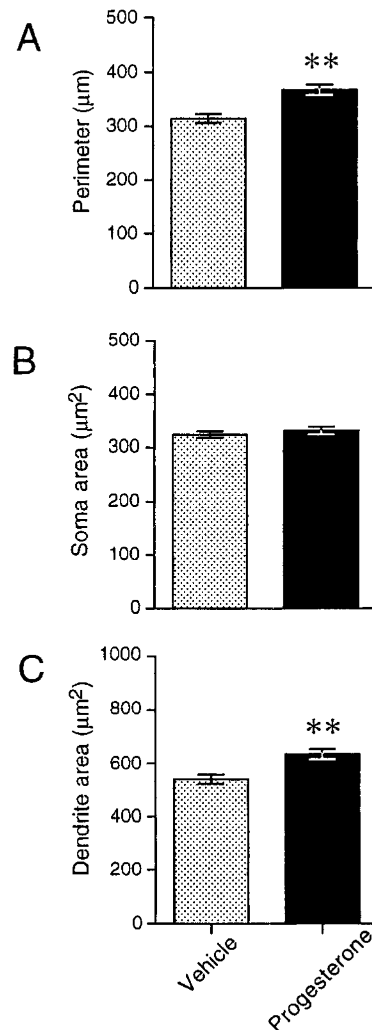

Figure 5. Morphological comparison of Purkinje cells from vehicle- and progesterone-treated groups: *in vivo* study. Perimeter (*A*), soma area (*B*), and dendrite area (*C*) of Purkinje cells (lobe IX) were measured after immunostaining for calbindin. Progesterone administration *in vivo* induced significant increases in the perimeter and dendrite area of Purkinje cells but not soma area. Each *column* and error bar represent the mean ± SEM ($n = 6$ males in each group). Data were derived from 120 Purkinje cells in each group. $^{**}p < 0.01$ (by Student's *t* test).

Expression of intranuclear receptors for progesterone in developing Purkinje cells

To demonstrate the cerebellar localization of PR, the expression of mRNA encoding for PR (isoforms type A and B) in the rat cerebellum was examined during neonatal development and in adults by RT-PCR analysis. One hundred nanograms of total RNA were extracted from the cerebellum of male and female rats of 0, 3, 7, 14, 21, and 60 d of age. The pituitary of the adult female rat was used as a positive control tissue, and the same amount of

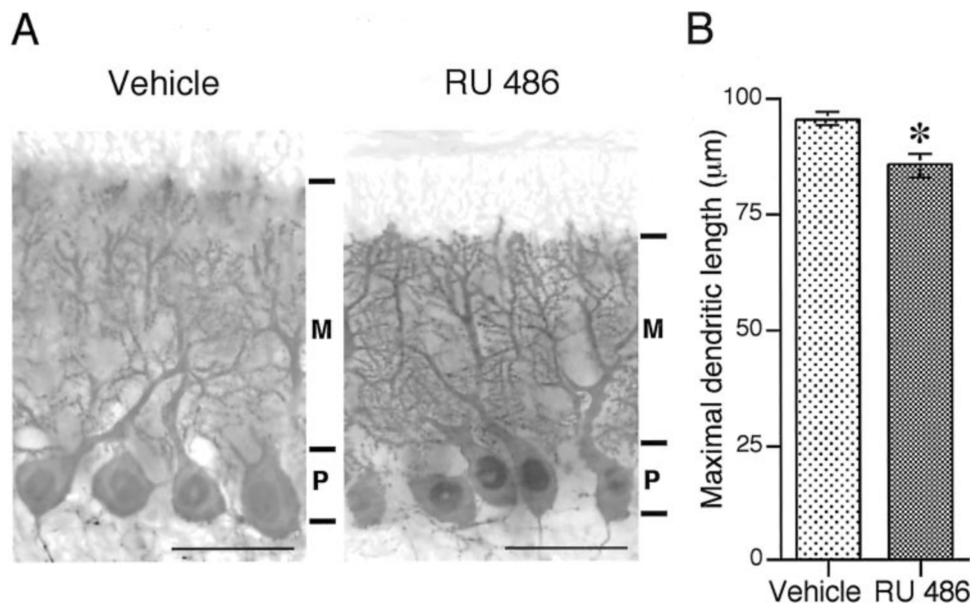

Figure 6. Morphological comparison of Purkinje cells from vehicle- and RU 486-treated groups: *in vivo* study. *A*, Parasagittal sections of neonatal cerebellum at 11 d of age were immunostained for calbindin (lobe IX). Male pups received daily injections of the vehicle (*left*) or RU 486 (*right*) for 4 d from 7–10 d old during the endogenous peak of progesterone in the cerebellum. Scale bars, 50 μm. *M*, Molecular layer; *P*, Purkinje cell layer. *B*, Quantitative analysis of the length of molecular layer as a parameter of maximal dendritic length. Dendritic outgrowth of the Purkinje cell was significantly inhibited by RU 486 administration. Each *column* and error bar represent the mean ± SEM (n = 4 males in each group). $^*p < 0.05$ (by Student's t test).

cDNA was used in the RT-PCR. The initial RNA amount used in the RT-PCR was adjusted spectrophotometrically. RT-PCR for β-actin was performed as a control experiment (Fig. 8*A*, *bottom panel*). Gel electrophoresis of the RT-PCR product for the PR (isoforms type A and B) gene identified a single band of 326 bp corresponding to PR mRNA size but not PR genomic DNA size in the cerebellum (Fig. 8*A*, *top panel*). Interestingly, cerebellar expression of the message was already detectable at 0 d of age and rapidly increased at 7 d of age (Fig. 8*A*, *top panel*). Expression tended to decrease at 14 d of age, followed by an increase thereafter, suggesting an age-dependent change of PR mRNA in both sexes (Fig. 8*A*, *top panel*). Serial Southern hybridization confirmed that this band was PR mRNA specific (Fig. 8*A*, *middle panel*).

Finally, cerebellar localization of PR was immunocytochemically examined with the antiserum raised against human PR (DNA-binding domain). PR-like immunoreactivity was present in the cerebellar cortex and restricted to the cell nucleus in both neonate (7 d of age) and adult (60 d of age) (Fig. 8*B*). As shown by *arrows* in Figure 8*B*, an intense immunoreaction for PR was observed in the large cell nuclei lying at a narrow zone between the molecular and granular layers at both 7 and 60 d of age. The distribution of immunoreactive cell nuclei in the cerebellar cortex was coincident with the location of nuclei of Purkinje cells, characterized by the Nissl staining (Fig. 8*B*). An immunoreaction with PR was observed only in Purkinje cell nuclei at 7 d of age (Fig. 8*B*). In contrast, PR-like immunoreactivity in the cerebella of adults was observed in relatively small cell nuclei (Fig. 8*B*, *arrowheads*) located in the molecular layer, as well as in Purkinje cell nuclei (Fig. 8*B*, *arrows*). Preadsorbing the antiserum with an excess amount of PR (DNA-binding domain; amino acids 533–547; 100 μg/ml) resulted in a complete absence of PR-like immunoreactivity in all of the positively stained cells in the cerebellum (Fig. 8*B*). Controls in which normal rabbit serum was substituted for the anti-PR serum also showed no immunoreactivity in the cerebellum. Together, localization of PR in the neonatal cerebellar cortex appears to be restricted to the Purkinje cell nuclei.

DISCUSSION

We have demonstrated recently that, in rats, the Purkinje cell, a cerebellar neuron, possesses the neurosteroidogenic enzymes cytochrome P450scc and 3β-HSD and produces pregnenolone, pregnenolone sulfate, and progesterone from cholesterol (Ukena et al., 1998, 1999a; Tsutsui et al., 2000). This is the first observation of neuronal neurosteroidogenesis in the mammalian brain. Interestingly, this neuron produced significant amounts of progesterone, as a product of an increase of 3β-HSD activity, only during a limited neonatal period, when cerebellar cortical formation occurs drastically (Altman, 1972a,b). Therefore, the aim of the present study was to clarify the organizing actions of progesterone on the growth and synaptic formation of Purkinje cells during cerebellar development. In this study, we also analyzed the action of 3α,5α-THP, a progesterone metabolite, which is also produced in the rat Purkinje cell during neonatal life (Tsutsui and Ukena, 2000; Tsutsui et al., 2000). *In vitro* treatment with progesterone using cerebellar slice cultures from newborn rats resulted in the promotion of dendritic outgrowth of Purkinje cells. This stimulatory effect occurred in a dose-dependent manner with a threshold concentration within the physiological range, i.e., 1–10 nM. However, there was no evidence for an effect of progesterone on Purkinje somata. These results were consistent with *in vivo* experiments in which progesterone administration to newborn rats induced dendritic outgrowth of Purkinje cells. Cerebellar progesterone concentration of newborn rats was negligible, whereas progesterone administration induced a significant increase in cerebellar progesterone level to a concentration similar to the maximal level observed in neonatal rats at 10 d of age under normal development (Ukena et al., 1999a). Thus, both *in vitro* and *in vivo* studies suggest that progesterone, produced as a neurosteroid in Purkinje cells during neonatal life, may be involved in the promotion of dendritic growth of the Purkinje cell. To investigate whether progesterone induces the promotion of axonal growth of Purkinje cells during development, additional morphological studies are now in progress.

The hypothesis postulated here that progesterone acts to promote dendritic growth of the Purkinje cell is supported by the present finding with the anti-progestin RU 486. The stimulatory action of progesterone on Purkinje cell dendrites was completely blocked by RU 486 *in vitro* by combined administration of progesterone and RU 486. In contrast, RU 486 alone failed to influence dendritic growth of the Purkinje cell, suggesting that

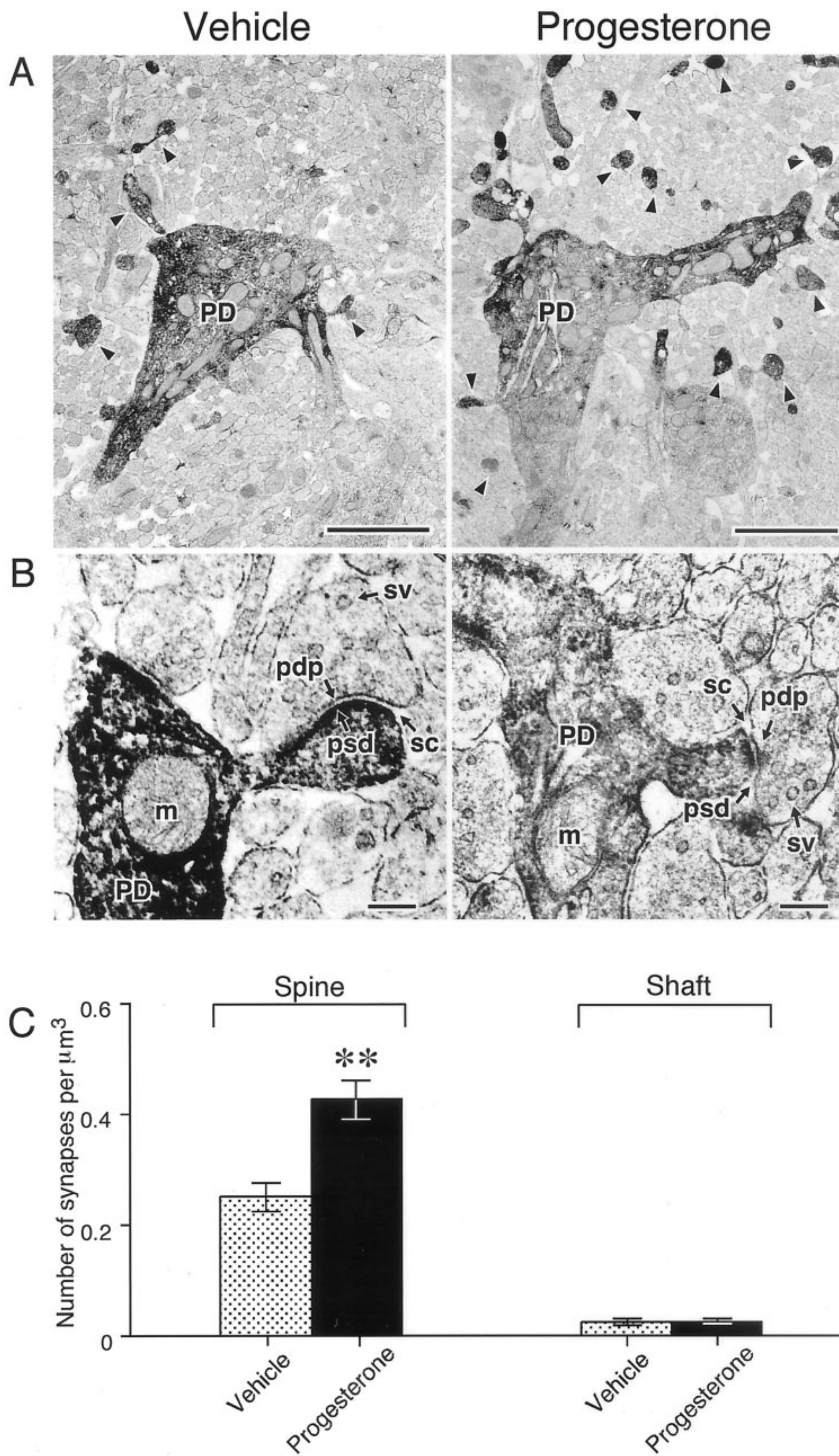

Figure 7. Ultrastructural analysis of Purkinje cells: *in vivo* study. *A*, Calbindin immunoelectron micrographs of Purkinje cell dendrites in the molecular layers of vermal cerebella (lobe IX). Spine number and synapse density were increased in progesterone-administered pups. *Arrowheads* in *A* indicate presumptive spine structures. *B*, Higher magnification of synaptic terminals in the molecular layers of vermal cerebella (lobe IX). The morphology of synaptic boutons and neurotransmitter vesicles appeared unaltered in progesterone-administered pups. *Arrows* in *B* indicate postsynaptic density (*psd*), synaptic vesicle (*sv*), synaptic cleft (*sc*), and presynaptic dense projections (*pdp*). *C*, Quantitative electron microscopic analysis using an unfolding method. Axospinous synapse density of Purkinje cells in the progesterone-treated group was significantly higher than in the control group (*vehicle*), unlike that of the density of dendritic shafts. Each *column* and error bar represent the mean ± SEM ($n = 6$ males in each group). Data were derived from randomly selected 24 fields (100 μm^2 in each field) of vermal molecular layers in each group. $^{**}p < 0.01$ (by Student's *t* test). *PD*, Purkinje cell dendrite; *m*, mitochondrion. Scale bars: *A*, 2 μm; *B*, 200 nm.

endogenous synthesis of progesterone in cultures of newborn rats was low in this study. Furthermore, *in vivo* administration of RU 486 during the endogenous peak of progesterone inhibited dendritic outgrowth of the Purkinje cell. To our knowledge, this is the first report showing cerebellar cortical organization by progesterone, produced as a neurosteroid in Purkinje cells. On the other hand, dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS) are also abundant neurosteroids in the mamma-

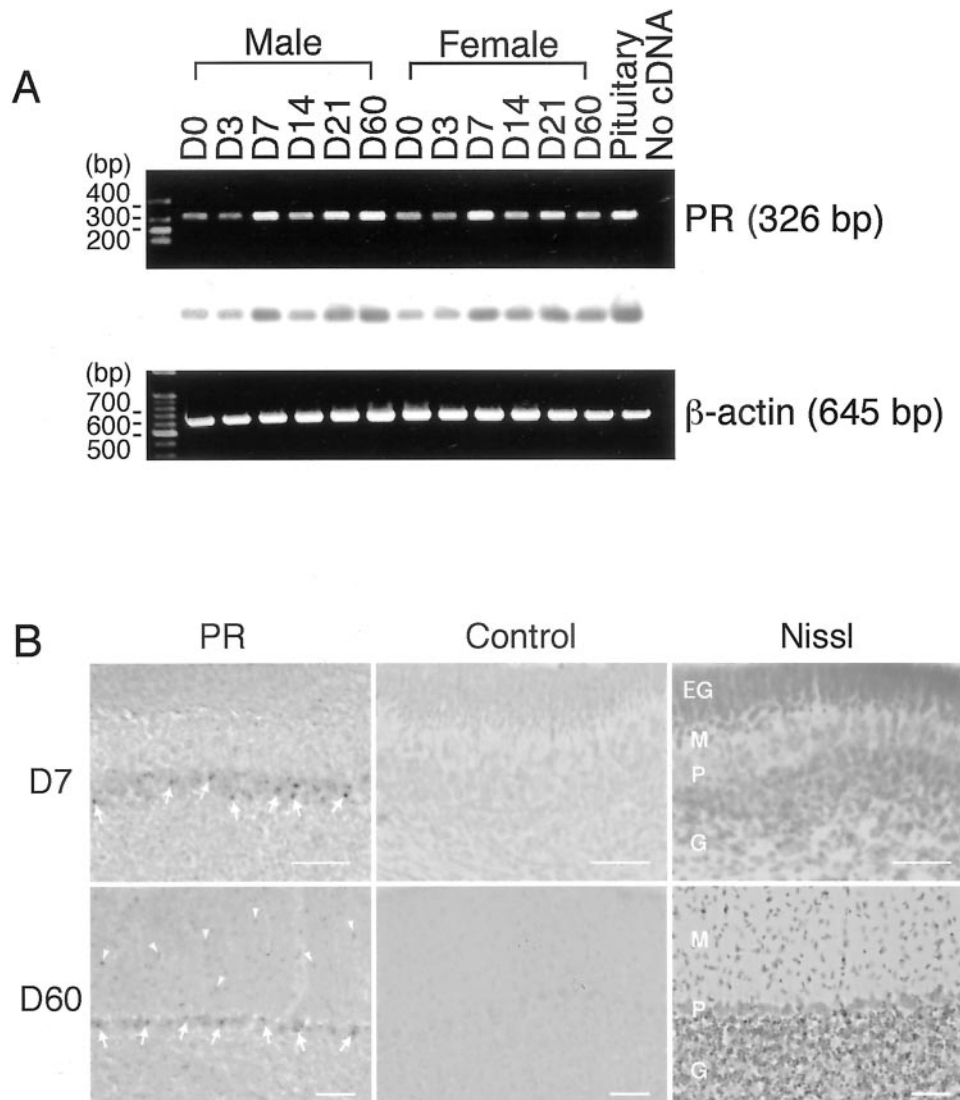

Figure 8. Expression and localization of progesterone receptor in the cerebellum: *in vivo* study. *A*, RT-PCR analysis of progesterone receptor mRNA in the male and female cerebella at 0, 3, 7, 14, 21, and 60 d of age. *Top panel* shows a result of gel electrophoresis of RT-PCR products for rat progesterone receptor, and *middle panel* shows an identification of the band by Southern hybridization using digoxigenin-labeled oligonucleotide probe for rat progesterone receptor. cDNA corresponding to 0.1 μg of total RNA extracted from each cerebellar tissue was used for a PCR reaction, and an 8 μl aliquot of each sample was applied on one lane. Pituitary tissue was used as a positive control, and a similar amount of cDNA was used in the RT-PCR. The *lane* labeled *No cDNA* was performed without template as the negative control. *Bottom panel* shows a result of the RT-PCR for β-actin as the internal control, in which PCR reaction, cDNA corresponding to 0.1 μg of total RNA, was used as a template. RT-PCR studies were repeated four times using independently extracted RNA samples from different animals and produced similar results. *B*, Immunocytochemical analysis using progesterone receptor antiserum (*left panels*; *PR*) of the cerebellar cortex of male rats at 7 d (*top panels*; *D7*) and 60 d of age (*bottom panels*; *D60*) (adult). An intense immunoreaction for progesterone receptor was observed in both groups in large cell nuclei lying in a narrow zone between the molecular and granular layers, possibly Purkinje cells (*arrows* in *PR*). In contrast, progesterone receptor-like immunoreactivity in the cerebella of adults was also observed in relatively small cell nuclei, possibly basket and/or satellite cells (*arrowheads* in *PR*). Preadsorbing the antiserum with an excess amount of the synthetic progesterone receptor peptide used as antigen (DNA-binding domain; amino acid 533–547; 100 μg/ml) resulted in a complete absence of progesterone receptor-like immunoreactivity in all of the positively stained cells in the cerebellum (*middle panels*; *Control*). Histology of the cerebellar cortex was revealed by Nissl staining (*right panels*; *Nissl*). Immunocytochemical studies were repeated independently five times using different animals and produced similar results. *EG*, External granular layer; *M*, molecular layer; *P*, Purkinje cell layer; *G*, granular layer. Scale bars, 50 μm.

lian brain (Corpéchot et al., 1981, 1983; Jo et al., 1989). Recently, Compagnone and Mellon (1998) reported a similar action of DHEA and DHEAS on neuronal growth using primary cultures of mouse embryonic neocortical neurons. According to Compagnone and Mellon (1998), DHEA selectively increased the length of axons and the incidence of varicosities and basket-like process formations *in vitro*, whereas DHEAS selectively promoted branching and dendritic outgrowth *in vitro*. Therefore, neurosteroids may play an important role in cortical organization in both the mammalian cerebellum and cerebrum during development.

Based on light and electron microscopic analyses, we further found that progesterone administration to newborn rats may induce an increase in the number of Purkinje dendritic spines. The most striking observation was the change in the density of dendritic axospinous synapses on Purkinje cells. In contrast, there was no significant change in the density of dendritic shaft synapses after progesterone administration. It is therefore possible that progesterone produced in neonatal Purkinje cells promotes not only dendritic growth but also spine synapse formation during cerebellar development. Estradiol-17β and progesterone are well known as important classical steroids secreted from the ovary, and several investigators have shown ovarian estradiol-mediated changes in dendritic spine synapse density but not in dendritic arborization in hippocampal CA1 pyramidal cells of adult female rats during the estrous cycle (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992, 1993; Woolley, 1998, 1999). In this study, progesterone alone induced not only dendritic arborization but also axospinous synapse formation in neonatal Purkinje cells. Notwithstanding such a discrepancy, this is the first observation of neurosteroid action on the promotion of synaptogenesis during neonatal life.

To understand the mode of organizing action of progesterone, the identification of PR in neonatal cerebellum is essential. There were no studies of PR expression during cerebellar development, although cerebellar expressions of PR type A and type B isoforms were only reported in adult female rats (Kato et al., 1993, 1994). The present RT-PCR analysis using common primers to isoforms type A and type B followed by Southern hybridization revealed that the expression of PR mRNA in the rat cerebellum increases during the neonatal period, i.e., 7 d of age. In addition, PR

expression was localized immunocytochemically in the Purkinje cell nucleus during neonatal life. Consequently, it is possible that progesterone acts directly on the Purkinje cell through intranuclear receptor-mediated mechanisms to promote the dendritic outgrowth and synaptogenesis in Purkinje cells during cerebellar cortical formation. PR mRNA was expressed in the cerebellum just after birth, i.e., 0–3 d of age, but the level was low. The developing gonad does not actively produce steroids until late neonatal life (Greco and Payne, 1994). Fetuses are exposed to maternal progesterone that readily crosses the placenta and is presumably present in mother's milk (Wagner et al., 1998). Maternal progesterone might also contribute to Purkinje cell development at approximately birth. In contrast, DHEA and DHEAS may exert their organizing actions via nonclassical steroid hormone receptors in the mouse embryonic neocortical neuron (Baulieu and Robel, 1998; Compagnone and Mellon, 1998). Such a difference in the mode of neurosteroid action in neurons may depend on the physicochemical properties of the steroid form. Additional study is required to draw a firm conclusion.

It is well known that drastic morphological changes occur in the rat cerebellum during neonatal life (Altman, 1972a,b). Purkinje cells differentiate at 3 d of age and develop markedly during neonatal life, concomitant with an increase in progesterone. The formation of the rat cerebellar cortex is almost complete at ~20 d of age. From these morphological findings (Altman, 1972a,b) together with recent our studies in the rat (Ukena et al., 1998, 1999a; Tsutsui and Ukena, 1999; Tsutsui et al., 2000), it can be supposed that progesterone produced by the Purkinje cell contributes to the formation of the cerebellar neuronal circuit via mechanisms that promote dendritic outgrowth and synaptogenesis in the Purkinje cell. Progesterone has also been shown to promote myelination in the peripheral nervous system via a classical steroid receptor (Koenig et al., 1995; Jung-Testas et al., 1999; Chan et al., 2000). These results in the peripheral nervous system are in agreement with the present findings in the CNS.

In contrast to progesterone, we could not detect any significant effects of $3\alpha,5\alpha$-THP, a progesterone metabolite, on Purkinje cell development. A number of studies using the patch-clamp method have indicated that $3\alpha,5\alpha$-THP mediates its action through ion-gated channel receptors, such as $GABA_A$, rather than through intracellular steroid receptors that promote classical genomic actions (for review, see Baulieu, 1997). Accordingly, it is probable that $3\alpha,5\alpha$-THP cannot bind to PR localized in the Purkinje cell, as suggested previously (MacDonald and Olsen, 1994; Baulieu, 1997), and consequently fails to induce Purkinje cell growth. However, Brinton (1994) has reported that $3\alpha,5\alpha$-THP may regulate nerve growth in rat cultured neurons. To establish the existence of any organizing action of $3\alpha,5\alpha$-THP in the cerebellum, therefore, it is necessary to analyze the effect of this steroid on other cerebellar cells during neonatal life.

The cerebellar Purkinje cell is a typical site for neurosteroid formation in rats (Ukena et al., 1998, 1999a; Tsutsui and Ukena, 1999; Tsutsui et al., 2000), as well as other vertebrates, including birds (Usui et al., 1995; Tsutsui et al., 1997a,b) and amphibians (Takase et al., 1999). This neuron produces significant amounts of progesterone from cholesterol, as a consequence of increased 3β-HSD activity occurring only during rat neonatal life. In conclusion, we have shown that, in rats, progesterone acts directly on Purkinje cells via intranuclear receptors to promote Purkinje dendritic growth and synaptogenesis during the neonatal period. Such a genomic action of progesterone may be essential for the formation of the cerebellar neuronal circuit that occurs during this period. Previously, we reported that pregnenolone sulfate, one of the neurosteroids synthesized in Purkinje cells, may modulate GABAergic transmission by nongenomic actions on GABAergic neurons rather than through genomic mechanisms (Tsutsui et al., 1997a, 2000; Tsutsui and Ukena, 1999). Because Purkinje cells play an important role in the process of memory and learning, they may serve as an excellent cellular model for the study of neurosteroid functions. Therefore, future attention should be focused on behavioral studies, as well as morphological and electrophysiological studies using steroidogenic enzyme and/or steroid receptor knock-out animals.

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