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Review article

Novel brain function: biosynthesis and actions of neurosteroids in neurons

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Abstract

Peripheral steroid hormones act on brain tissues through intracellular receptor-mediated mechanisms to regulate several important brain neuronal functions. Therefore, the brain is considered to be a target site of steroid hormones. However, it is now established that the brain itself also synthesizes steroids de novo from cholesterol. The pioneering discovery of Baulieu and his colleagues, using mammals, and our studies with non-mammals have opened the door of a new research field. Such steroids synthesized in the brain are called neurosteroids. Because certain structures in vertebrate brains have the capacity to produce neurosteroids, identification of neurosteroidogenic cells in the brain is essential to understand the physiological role of neurosteroids in brain functions. Glial cells are generally accepted to be the major site for neurosteroid formation, but the concept of neurosteroidogenesis in brain neurons has up to now been uncertain. We recently demonstrated neuronal neurosteroidogenesis in the brain and indicated that the Purkinje cell, a typical cerebellar neuron, actively synthesizes several neurosteroids de novo from cholesterol in both mammals and non-mammals. Pregnenolone sulfate, one of neurosteroids synthesized in the Purkinje neuron, may contribute to some important events in the cerebellum by modulating neurotransmission. Progesterone, produced as a neurosteroid in this neuron only during neonatal life, may be involved in the promotion of neuronal and glial growth and neuronal synaptic contact in the cerebellum. More recently, biosynthesis and actions of neurosteroids in pyramidal neurons of the hippocampus were also demonstrated. These serve an excellent model for the study of physiological roles of neurosteroids in the brain, because both cerebellar Purkinje neurons and hippocampal neurons play an important role in memory and learning. This paper summarizes the advances made in our understanding of neurosteroids, produced in neurons, and their actions. © 2000 Elsevier Science Ireland Ltd and the Japan Neuroscience Society. All rights reserved.

Keywords: Neurosteroids; Pregnenolone; Pregnenolone sulfate; Progesterone; Cytochrome P450 side-chain cleavage enzyme; 3β-Hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase; Purkinje neurons; Hippocampal neurons; Neurotransmission; Neuronal circuit

1. Introduction to a new research field

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 (Fuxe et al., 1981; McEwen, 1991). Gonadal androgens, for instance, act on the brain to influence several reproductive behaviors in vertebrates. Many of the brain regions that control a variety of reproductive behaviors contain a high proportion of cells that concentrate androgenic hormones. Therefore, the brain is considered to be a target site of peripheral steroids. In addition to direct steroidal actions, the metabolism of peripheral steroids in brain tissues can result in biotransformation and the production of biologically active metabolites. Indeed,

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androgenic action in the vertebrate brain is often mediated by the enzymatic activity of cytochrome P450arom which catalyzes the conversion of androgen to estrogen. Both P450arom and estrogen receptors are expressed in several brain regions, including the hypothalamus and preoptic area, which are involved in the control of reproductive behaviors.

On the other hand, new findings have been obtained that the nervous system itself may form steroids de novo. The pioneering discovery of Baulieu and his colleagues, using rodents, has opened the door of a new research field for many laboratories. Pregnenolone[1] and dehydroepiandrosterone, as unconjugated steroids, and their fatty acid or sulfate esters, accumulate within the brains of several mammalian species (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Lanthier and Patwardhan, 1986; Robel et al., 1986, 1987; Jo et al., 1989; Mathur et al., 1993). 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 brain can synthesize steroids de novo (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Robel et al., 1986, 1987; Jo et al., 1989). In contrast to extensive mammalian studies, especially using rodents, little has been known regarding de novo steroidogenesis in the brain of non-mammalian vertebrates. We therefore looked for steroids formed from cholesterol in the brains of both avian (Tsutsui and Yamazaki, 1995; Usui et al., 1995, Tsutsui et al., 1997a, b) and amphibian species (Takase et al., 1999). Independently, other groups, such as Vaudry's laboratory (Mensah-Nyagan et al., 1994) and Schlinger's laboratory (Vanson et al., 1996), also contributed to this area. The formation of several steroids from cholesterol is now known to occur in non-mammalian vertebrates. Such steroids synthesized in vertebrate brains are called neurosteroids. Our recent studies have focused on investigating physiological changes in neurosteroids in the brain of seasonally breeding vertebrates (Clark et al., 1999; Takase et al., 1999), as this would contribute to our understanding of neurosteroid action.

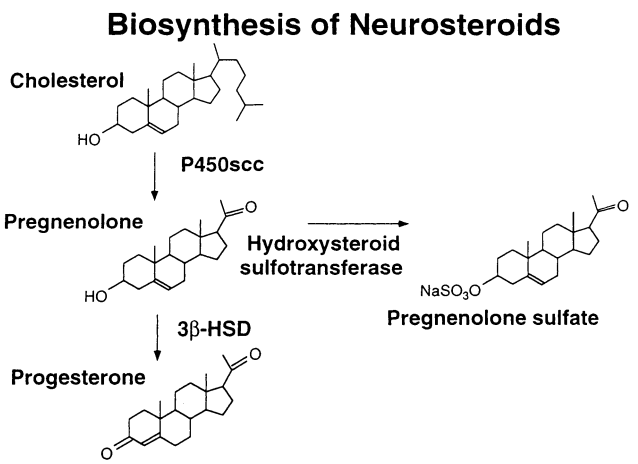

Fig. 1. Biosynthetic pathway for neurosteroids which is identified in cerebellar Purkinje neurons. P450scc, cytochrome P450 side-chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase.

When we understand the physiological role of neurosteroids in brain function, it is also essential to identify the cells involved in neurosteroidogenesis. In recent years knowledge has been accumulated in mammals that glial cells play a major role in neurosteroid formation and metabolism in the brain. Both oligodendrocytes and astrocytes are considered to be the primary site for pregnenolone synthesis, an initial step of neurosteroidogenesis. However, whether neurons located in the brain produce neurosteroids has remained unclear. With these findings as a background, we have demonstrated the presence and activity of neurosteroidogenic enzymes in a cerebellar neuron. Interestingly, the Purkinje cell possesses neurosteroidogenic enzymes and produces neurosteroids de novo in a variety of vertebrates including mammalian species (Usui et al., 1995, Tsutsui et al., 1997a, b, Ukena et al., 1998, 1999a, Takase et al., 1999). This is the first demonstration of neuronal de novo neurosteroidogenesis in the brain and serves an excellent model for the study of physiological roles of neurosteroids in the brain. More recently, neurosteroid formation in the hippocampal pyramidal neuron was also demonstrated in mammals (Kawato, 2000).

In this review, we summarize the advances made in our understanding of neurosteroid biosynthesis, including neuronal neurosteroidogenesis, in vertebrate brains by our recent studies and the related studies of other laboratories. These also include possible actions of neurosteroids produced in neurons. For detailed information of neurosteroids in glial cells the reader is referred to an excellent review (Baulieu, 1997).

[1] Pregnenolone, a 3β-hydroxy-Δ^5-steroid, is the main precursor of various steroid hormones produced in peripheral steroidogenic glands and formed from cholesterol by the oxidative side-chain cleavage reaction, which is catalyzed by a specific enzyme, cytochrome P450 side-chain cleavage enzyme (cytochrome P450scc). 3β-Hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3ß-HSD) is also a key enzyme for steroidogenesis in peripheral steroidogenic glands. The enzyme 3ß-HSD catalyzes the oxidation and isomerization of 3β-hydroxy-Δ^5-steroids, such as pregnenolone and dehydroepiandrosterone. The enzyme 17α-hydroxylase/c17,20-lyase (cytochrome $P450_{17\alpha,lyase}$) converts pregnenolone to dehydroepiandrosterone. In addition to peripheral steroidogenic glands, the expressions of these key enzymes in the brain were recently demonstrated in mammalian and non-mammalian vertebrates. Steroids synthesized from cholesterol in the brain as well as other nervous systems are called neurosteroids. Purkinje cell, a typical cerebellar neuron, possesses cytochrome P450scc and 3ß-HSD and actively synthesizes several neurosteroids de novo from cholesterol (See Fig. 1). This is the first demonstration of neuronal neurosteroidogenesis in the brain.

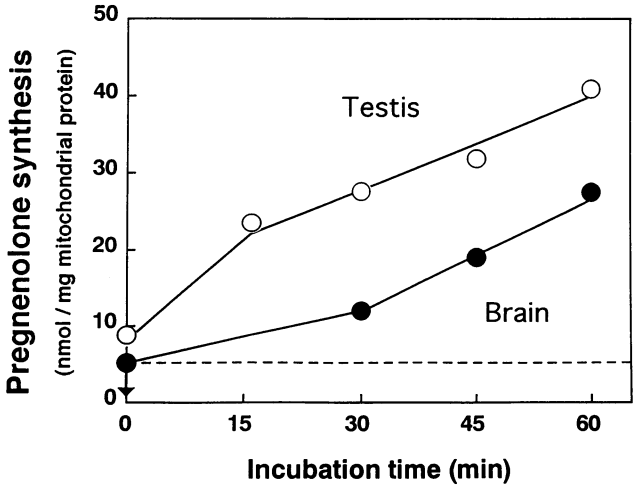

Fig. 2. Pregnenolone biosynthesis from cholesterol in intact mitochondria of the whole brain and the testis of adult male quails. Incubations were performed with 200 μM cholesterol, 0.25 mg of mitochondrial protein, 10 mM isocitrate and 10 mM succinate at 41°C for different times. See (Tsutsui and Yamazaki, 1995) for details.

2. Vertebrate brains produce neurosteroids

2.1. Mammals

Pregnenolone, a 3β-hydroxy-Δ^5-steroid, is a main precursor of steroid hormones secreted by steroidogenic glandular cells. 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. Therefore, it is essential to demonstrate the formation of pregnenolone in the brain (see Fig. 1). A number of studies with several species of mammals have reported that the brain contains abundant quantities of 3β-hydroxy-Δ^5-steroids, i.e. pregnenolone, dehydroepiandrosterone and their fatty acid or sulfate esters. Furthermore, the content of these steroids in the brain is virtually constant even after the removal of peripheral steroids (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Lanthier and Patwardhan, 1986; Robel et al., 1986, 1987; Jo et al., 1989; Mathur et al., 1993; Ukena et al., 1998). It has also been demonstrated that certain structures in the mammalian brain possess the P450scc enzyme (Hu et al., 1987; Le Goascogne et al., 1987; Jung-Testas et al., 1989; Iwahashi et al., 1990; Papadopoulos et al., 1992; Mellon and Deschepper, 1993; Compagnone et al., 1995a; Ukena et al., 1998). Recent studies have further demonstrated that both P450scc protein and its messenger RNA (mRNA) are expressed in the rat brain (Jung-Testas et al., 1989; Baulieu and Robel, 1990; Baulieu, 1991; Mellon and Deschepper, 1993; Compagnone et al., 1995a; Kohchi et al., 1998; Ukena et al., 1998). Thus, the formation of pregnenolone and its ester from cholesterol is well established in the mammalian brain. In contrast, the formation of dehydroepiandrosterone and its ester in the brain remains to be clarified, even though glial cells isolated from neonatal rat brains may be able to convert pregnenolone to dehydroepiandrosterone (Zwain and Yen, 1999). Progesterone, a 3-oxo-Δ^4-steroid, and its metabolites, such as 3α-dihydroxy-progesterone and 3α,5α-tetrahydroxy-progesterone, are also produced and accumulate in the mammalian brain as neurosteroids (see Fig. 1). The biosynthesis of progesterone is performed by 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD) which catalyzes the dehydrogenation and isomerization of the Δ^5-3β-hydroxysteroids (pregnenolone and dehydroepiandrosterone) into Δ^4-ketosteroids (progesterone and androstenedione, respectively) and is highly expressed in the classical steroidogenic glands (Mason, 1993). In several brain regions, the expression of both 3β-HSD protein and its mRNA has been reported in mammals (Dupont et al., 1994; Guennoun et al., 1995; Sanne and Krueger, 1995; Kohchi et al., 1998; Ukena et al., 1999a). In addition, 3β-HSD activity has been demonstrated biochemically in mammalian brain tissues and cultured cells (Weidenfeld et al., 1980; Akwa et al., 1993; Kabbadj et al., 1993; Ukena et al., 1999a).

2.2. Non-mammals

In contrast to mammals, de novo steroidogenesis from cholesterol in the brain of non-mammalian vertebrates had not been documented until the first half of the 1990s. As an initial step in the demonstration of pregnenolone biosynthesis in the avian brain, Tsutsui and Yamazaki (1995) measured the concentrations of pregnenolone and its sulfate ester in the whole brain of adult quails using a specific radioimmunoassay (RIA). Interestingly, the pregnenolone concentration in adult birds was much higher in the brain than in plasma. Hypophysectomy greatly reduced plasma pregnenolone concentrations, but a high level of pregnenolone persisted in the hypophysectomized birds suggesting that the accumulation of pregnenolone in the quail brain may be largely independent of peripheral steroidogenic glands. Pregnenolone sulfate ester was also detectable in the avian brain. Subsequently, Tsutsui and Yamazaki (1995) investigated the biochemical analysis of cytochrome P450scc activity. The formation of pregnenolone from cholesterol was assessed in intact mitochondria derived from the quail brain and compared with the formation of pregnenolone by testicular mitochondria. As shown in Fig. 2, pregnenolone was formed in brain mitochondria and the pregnenolone concentration per unit mitochondria protein increased as a function of incubation time thus suggesting that the side-chain cleavage of cholesterol is likely to occur

in quail brain mitochondria. To investigate the presence of cytochrome P450scc in the quail brain, Tsutsui and Yamazaki (1995) carried out Western immunoblot analysis with an antibody against purified bovine P450scc after SDS-gel electrophoresis of brain homogenates. In both the brain and testis, the antibody against P450scc predominantly recognized a protein band of electrophoretic mobility in the proximity of bovine P450scc. A similar result was obtained in the dove and finch brains (unpublished observations). Taken together, these biochemical and immunochemical studies indicate that the avian brain possesses cytochrome P450scc and produces pregnenolone from cholesterol (for reviews, see Tsutsui et al., 1997a, b).

Subsequently we have extended our understanding of pregnenolone biosynthesis in the brains of lower vertebrates. Takase et al. (1999) demonstrated that the amphibian brain possesses P450scc and produces pregnenolone and its sulfate ester. According to Takase et al. (1999), the concentrations of pregnenolone and its sulfate ester in the brain of *Xenopus laevis* were greater than that in the gonads and plasma. An immunoreactive protein band of electrophoretic mobility in the proximity of bovine P450scc was detected in the *Xenopus* brain as well as the gonads by Western blot analysis. As a lower vertebrate species also possesses P450scc in the brain, the presence of P450scc may thus be considered as a conserved property of vertebrate brains.

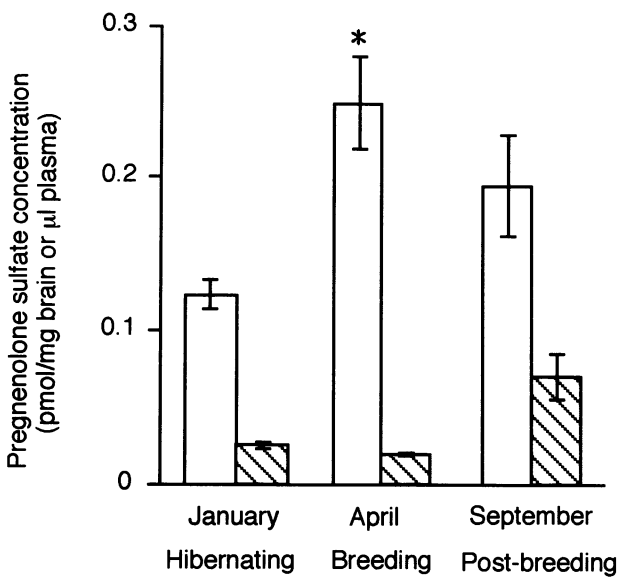

Fig. 3. Seasonal changes in pregnenolone sulfate concentrations in the brain (open column) and plasma (shaded column) from adult female *Rana nigromaculata*. Frogs were killed in April (breeding phase), September (post-breeding phase), and January (hibernating phase). Each column and vertical line represent the mean ± SEM ($n = 5$ samples in each phase; one sample from one frog). Significance of difference: *, $P < 0.05$ versus January, by Duncan's multiple range test. A similar result obtained in adult male frogs. See (Takase et al., 1999) for details.

This is also true for the presence of 3β-HSD, because 3β-HSD activity has also been found in the brain of both avian (Vanson et al., 1996; Pignataro et al., 1998; Ukena et al., 1999b) and amphibian species (Mensah-Nyagan et al., 1994). Recently, Ukena et al. (1999b) demonstrated the expression of 3β-HSD mRNA in the avian brain. Thus, it is now established that de novo steroidogenesis occurs in vertebrate brains from cholesterol (for a review see Tsutsui et al., 1999). These steroids synthesized de novo from cholesterol in the brain as well as other areas of the nervous system are called neurosteroids.

2.3. Seasonal breeders

As mentioned above, de novo steroidogenesis from cholesterol appears in the brain of several vertebrates, such as mammals, birds and amphibians. Physiological changes in neurosteroid levels must be taken into account when understanding the function of neurosteroids in the vertebrate brain. If neurosteroids are involved in some important brain functions, it would be expected that they would change under different physiological conditions. To test this hypothesis, wild animal species may serve as excellent models. In contrast to the laboratory and domestic animals, the reproductive activity of most species of wild animals inhabiting the temperate and subtropical zones demonstrates a seasonal variation. Such a variation is the consequence of interaction between external environmental and internal factors. In wild animals, reproductive activity is usually confined to a short breeding period. At the termination of the breeding phase, the reproductive system undergoes regression and remains quiescent until the approach of next breeding phase. Puberty in young individuals generally coincides with the onset of the breeding phase.

We examined seasonal changes in the concentrations of pregnenolone and its sulfate ester in the brain of *Rana nigromaculata*, a seasonally breeding amphibian (Takase et al., 1999). The annual breeding cycle of this amphibian is divided into a hibernating phase (November–March), the breeding phase (April–May), and the post-breeding phase (June–October). Pregnenolone sulfate concentrations in the *Rana* brain were high during the active seasons, i.e. breeding phase (female) and post-breeding phase (male), and low during the quiescent season, i.e. hibernating phase (both sexes); whereas plasma pregnenolone concentrations were virtually constant throughout the year (Fig. 3) (Takase et al., 1999). In addition, such a seasonal change in pregnenolone sulfate observed in the brain may be independent of peripheral steroidogenic glands, because the change in the concentration of plasma pregnenolone sulfate was significantly different from that in the brain (Fig. 3).

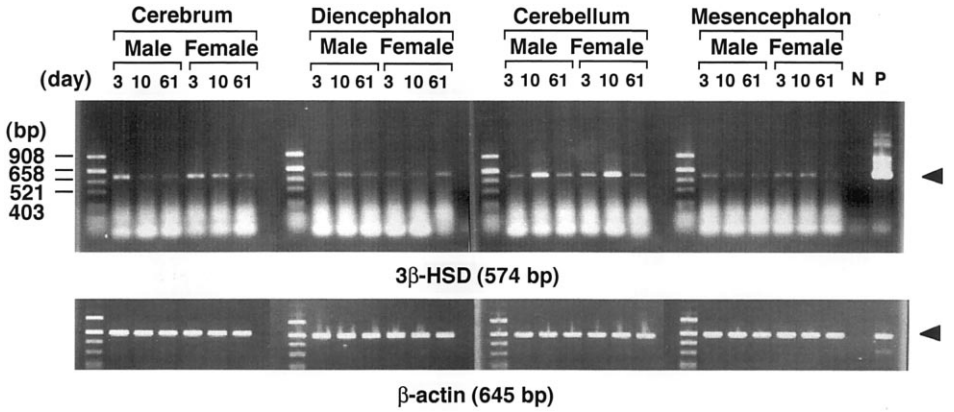

Fig. 4. RT-PCR analysis of the mRNA encoding for 3β-HSD in the rat brain during neonatal life and in adulthood. Total RNAs were extracted from cerebrum, diencephalon, cerebellum and mesencephalon of male and female rats at the ages of 3, 10 and 61 days, and used for RT-PCR analysis. RT-PCR products for rat 3β-HSD were examined by gel electrophoresis on 1.5% agarose gel. Lower panel shows a result of 1.5% agarose gel electrophoresis of the RT-PCR product for β-actin as the internal control. Total RNA amounts used in the experiment were 400 ng for 3β-HSD and 0.08 ng for β-actin per one lane. The arrowhead indicates 3β-HSD band (upper panel) or β-actin band (lower panel). The lane labeled 'N' was performed without template as the negative control, and the lane labeled 'P' was performed using ovary RNA as the positive control. Serial Southern hybridization in the experiment confirmed the specific 3β-HSD mRNA expression. See (Kohchi et al., 1998) for details.

3. Neurosteroidogenic enzymes in the brain and their changes during development

To clarify the biosynthetic and metabolic pathways of neurosteroids in the brain, extensive studies with several vertebrates, especially mammals, have been carried out by many laboratories. As indicated above, the presence of cytochrome P450scc and 3β-HSD has been well established in the vertebrate brain, whereas limited information has been available for the enzyme 17α-hydroxylase/c17,20-lyase (cytochrome $P450_{17\alpha,lyase}$), which converts pregnenolone to dehydroepiandrosterone, one of the most abundant neurosteroids in the brain. Therefore, Kohchi et al. (1998) investigated expression of the mRNAs encoding for three key steroidogenic enzymes, i.e. cytochrome P450scc, 3β-HSD and cytochrome $P450_{17\alpha,lyase}$, using rats at different postnatal ages in order to characterize the biosynthetic pathway of abundant neurosteroids, such as 3β-hydroxy-Δ^5-steroids and 3-oxo-Δ^4-steroids, in the brain from cholesterol. The expression of P450scc mRNA occurred throughout the brain at a similar level, while 3β-HSD mRNA expression was higher in the cerebellum and cerebrum than in other brain regions (Fig. 4) (Kohchi et al., 1998). Interestingly, the $P450_{17\alpha,lyase}$ mRNA was highly expressed in the mesencephalon (Kohchi et al., 1998). Higher expression of the cerebellar and cerebral 3β-HSD mRNAs was observed only in neonatal life (Fig. 4), but the expression of both P450scc mRNA and $P450_{17\alpha,lyase}$ mRNA was relatively constant during neonatal life and in adulthood (Kohchi et al., 1998). These results indicate that in the postnatal rat, the expression of 3β-HSD or $P450_{17\alpha,lyase}$ mRNA may be age- or region-dependent, unlike P450scc mRNA expression. Although other investigators (Guennoun et al., 1995; Sanne and Krueger, 1995) also demonstrated 3β-HSD mRNA expression in the rat brain, a pattern of age-related changes in brain 3β-HSD mRNA expression has not previously been reported in any mammalian species. Recently, an age-dependent expression of 3β-HSD in the cerebellum was confirmed by both biochemical and HPLC analysis (Ukena et al., 1999a). According to Ukena et al. (1999a), 3β-HSD activity in the cerebellum also increases during neonatal life. A great expression of cerebellar and cerebral 3β-HSD mRNAs during neonatal life suggests some functional role for the products (progesterone and its metabolites) of 3β-HSD activity, such as possibly the promotion of axonal growth of neurons and the synaptic contact of neurons.

As for $P450_{17\alpha,lyase}$, Strömstedt and Waterman (1995) also found, using RT-PCR analysis followed by Southern blots, a higher expression of the $P450_{17\alpha,lyase}$ mRNA in the brain stem of postnatal rats and mice. In addition, Compagnone et al. (1995b) reported that rat embryo cells expressing $P450_{17\alpha,lyase}$ are located in the mesencephalic region as well as the medulla and spinal cord. Since the level of 3β-HSD mRNA expression is low in the mesencephalon, dehydroepiandrosterone but not progesterone, 17α-hydroxy-progesterone and androstenedione may be produced as a principal neurosteroid in this brain region. More recently, Zwain and Yen (1999) reported that dehydroepiandrosterone may be biosynthesized in cultured glial cells isolated from neonatal rat brains by a $P450_{17\alpha,lyase}$-dependent mechanism. To draw a firm conclusion concerning $P450_{17\alpha,lyase}$ expression, however, further study is needed.

4. Neurosteroidogenic cells identified in the brain

4.1. Glial cells

To determine the localization of the steroidogenic enzyme P450scc in the brain, immunohistochemical analysis with the antibody against P450scc has been conducted in several vertebrate species. In the first immunohistochemical description of cytochrome P450scc by Le Goascogne et al. (1987), 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 (Hu et al., 1987). 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 immunohistochemically 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 (Hu et al., 1987; Jung-Testas et al., 1989; Baulieu and Robel, 1990; Akwa et al., 1991; Baulieu, 1991; Papadopoulos et al., 1992).

4.2. Purkinje neurons

In contrast to glial cells, the concept of neurosteroidogenesis in neurons in the brain has been uncertain. We have recently found the cerebellar neuron to be an active neurosteroidogenic cell, which possesses both P450scc and 3β-HSD and produces pregnenolone, pregnenolone sulfate and progesterone, in several vertebrate species (Usui et al., 1995 Tsutsui et al., 1997a, b Ukena et al., 1998, 1999a; Takase et al., 1999). Thus, our studies provided the first evidence for the location of P450scc and 3β-HSD in Purkinje cells and gave the opportunity to understand neuronal neurosteroidogenesis in the brain.

In our immunohistochemical studies of the quail brain using an antibody against P450scc, the most striking observation was the distribution of immunoreactive cells in the cerebellar cortex, although other immunopositive cells were detected in telencephalic and diencephalic regions (Usui et al., 1995, Tsutsui et al., 1997a, b). The distribution of immunoreactive cell bodies and fibers in the cerebellar cortex was coincident with the location of somata and dendrites of Purkinje cells (Usui et al., 1995, Tsutsui et al., 1997a, b). Western immunoblot analysis confirmed the presence of P450scc-like protein in the immunohistochemically stained cerebellar cortex (Usui et al., 1995). To the best of our knowledge, 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. However, whether neurons located in the mammalian brain possess cytochrome P450scc and produce pregnenolone and its sulfate ester still remained unclear. Therefore, we subsequently investigated the presence of P450scc in the cerebellar Purkinje cell using a mammalian species (Ukena et al., 1998). Immunohistochemical analysis demonstrated that immunoreaction with P450scc was contained to the somata and dendrites of Purkinje cells in the rat cerebellum (Fig. 5) (Ukena et al., 1998). An antibody against inositol triphosphate (IP_3) receptor, a marker of the Purkinje cell, recognized P450scc-immunoreactive cerebellar cells that showed no immunoreaction with glial fibrillary acidic protein (GFAP), a specific marker of glial cells (Fig. 5) (Ukena et al., 1998). In addition, the expressions of both P450scc-like protein and P450scc mRNA were detected in the rat cerebellum (Ukena et al., 1998). Thus, we may conclude that the rat Purkinje cell also possesses P450scc. Furthermore, in this mammalian species, P450scc appears in the Purkinje cell immediately after its differentiation and the expression of this enzyme persists during neonatal development into adulthood (Fig. 6) (Ukena et al., 1998).

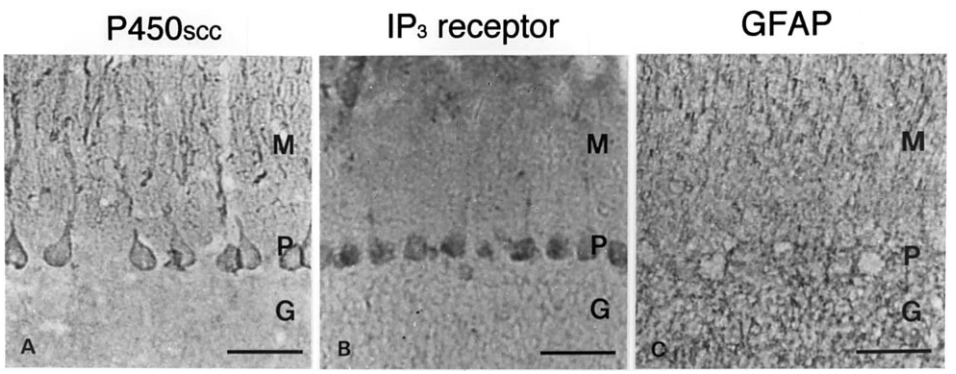

Fig. 5. Immunohistochemical staining with the antiserum to cytochrome P450scc (A), IP_3 receptor (B), or GFAP (C) in the cerebellar cortex of adult male rats. The antiserum to IP_3 receptor (B) or to GFAP (C) was used as a specific marker of Purkinje cells or glial cells. P, Purkinje cell layer; M, molecular layer; G, granular layer in the cerebellar cortex. Bars, 50 μm. See (Ukena et al., 1998) for details.

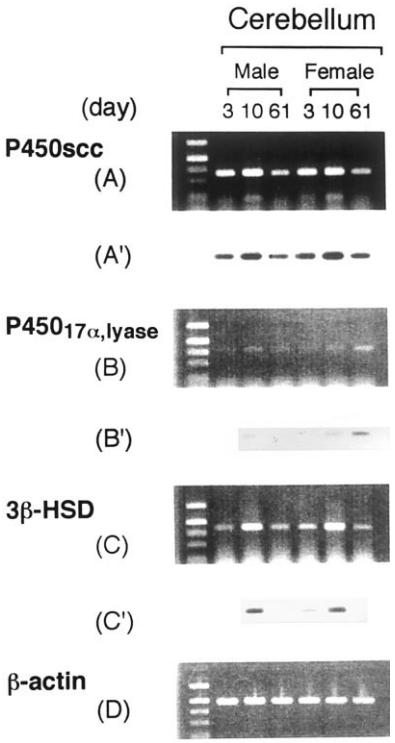

Fig. 6. RT-PCR analysis together with Southern hybridization of mRNAs encoding for P450scc, P450$_{17\alpha,lyase}$ and 3β-HSD in the rat cerebellum during neonatal life and in adulthood. Total RNAs were extracted from cerebella of male and female rats at the ages of 3, 10 and 61 days, and used for RT-PCR analysis. RT-PCR products for P450scc (A), P450$_{17\alpha,lyase}$ (B) and 3β-HSD (C) were examined by gel electrophoresis and serial Southern hybridization was performed with each digoxigenin-labeled oligonucleotide probe for P450scc (A′), P450$_{17\alpha,lyase}$ (B′) and 3β-HSD (C'). Lowest panel (D) shows a result of the RT-PCR for β-actin as the internal control. See (Ukena et al., 1998; Kohchi et al., 1998; Ukena et al., 1999a) for details.

In addition to higher vertebrates, our recent studies with amphibians further identified the presence of P450scc-like protein in the cerebellar Purkinje cell as well as other telencephalic and diencephalic cells of *Xenopus laevis* and *Rana nigromaculata* (Takase et al., 1999). Taken together, our findings obtained in both higher and lower vertebrates (Usui et al., 1995, Tsutsui et al., 1997a, b, Ukena et al., 1998; Takase et al., 1999) suggest that Purkinje cells possess P450scc and produce pregnenolone and its sulfate ester. To draw a firm conclusion, however, we need to demonstrate P450scc expression in Purkinje cells of other lower vertebrates.

It was more recently found that the steroidogenic acute regulatory protein (StAR) is expressed in Purkinje cells (Furukawa et al., 1998). 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 addition to cytochrome P450scc, we have demonstrated the expression of 3β-HSD and its enzymatic activity in the cerebellum of neonatal and adult rats using RT-PCR (Fig. 6) and biochemical analyses (Fig. 7). Subsequently, using in situ hybridization of 3β-HSD mRNA, the site of 3β-HSD expression was localized in Purkinje cells (Fig. 8) and external granule cells (Ukena et al., 1999a). These results indicate that 3β-HSD as well as P450scc are expressed in Purkinje cells. The expression of 3β-HSD, however, increased during the neonatal period, unlike P450scc (Fig. 6) (Ukena et al., 1999a). In addition, Purkinje cells during neonatal life produced not only progesterone but also its metabolite(s) (Tsutsui and Ukena, 2000). Such an age-dependent expression of 3β-HSD was confirmed by

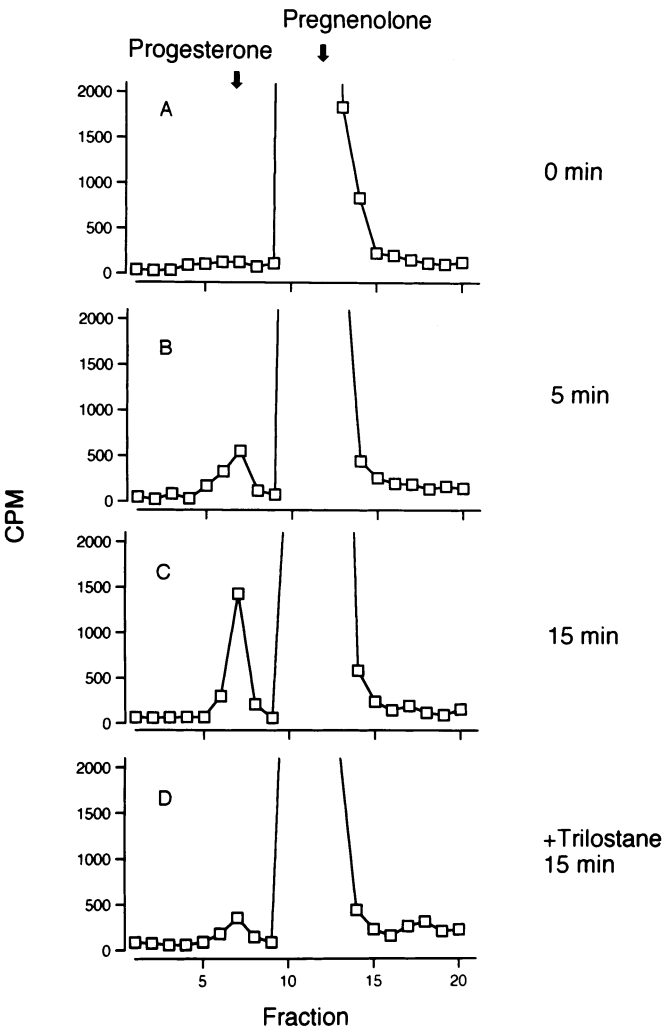

Fig. 7. HPLC analysis of steroids extracted from cerebellar slices of rats at 10 days of age after different incubation times (0 min (A), 5 min (B), and 15 min (C)) with tritiated pregnenolone using a reversed phase column. The column was eluted with an isocratic elution of 70% acetonitrile. The eluate was fractionated every 0.5 min from 10–20 min (0.35 ml each). The ordinate indicates radioactivity measured in each HPLC fraction. The arrows indicate the elution positions of progesterone and pregnenolone, respectively. The cerebellar slices were also incubated with 10^{-4} M trilostane, a specific inhibitor of 3β-HSD, for 15 min and subjected to HPLC analysis (D). See (Ukena et al., 1999a) for details.

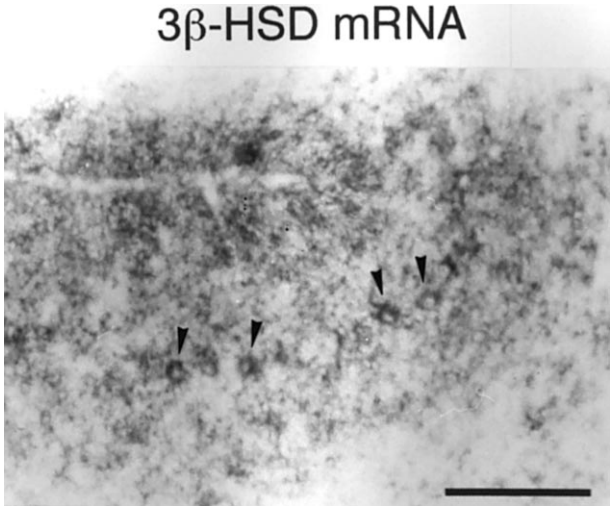

Fig. 8. In situ hybridization using digoxigenin-labeled oligonucleotide probes for 3β-HSD in the cerebellar cortex of neonatal rats at 10 days of age. The arrowheads show Purkinje cells expressing 3β-HSD mRNA. Bar, 100 μm. See (Ukena et al., 1999a) for details.

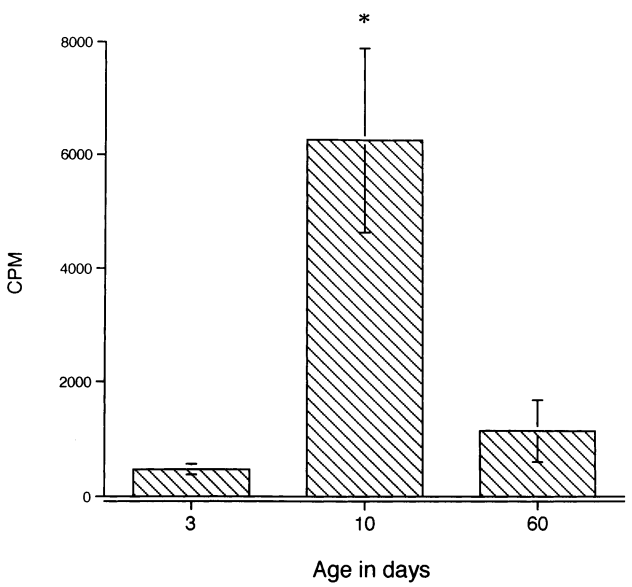

Fig. 9. Comparison of 3β-HSD enzymatic activity among rat cerebella at 3, 10 and 60 days of age. Cerebellar slices adjusted to the same weight were incubated with tritiated pregnenolone for 15 min and subjected to HPLC analysis. Each column and vertical line represent the mean ± SEM radioactivity corresponding to progesterone ($n = 4$ samples). Significance of difference: *, $P < 0.05$ versus 3 and 60 days, by Duncan's multiple range test. See (Ukena et al., 1999a) for details.

biochemical studies together with HPLC analysis, indicating an increase of progesterone formation during neonatal life (Fig. 9) (Ukena et al., 1999a). Notwithstanding such a difference in the age-dependent expression, a number of our studies have indicated that the Purkinje cell, a typical cerebellar neuron, is an important neurosteroidogenic cell in the vertebrate brain (Fig. 10) (for a review, see Tsutsui and Ukena, 1999).

4.3. Other neurons

In addition to Purkinje neurons, the localization of neurosteroidogenic enzymes in other neurons has recently characterized (Kawato, 2000). In the rat hippocampus, significant localization was observed for P450scc, $P450_{17\alpha,lyase}$, and P450arom in pyramidal neurons in the CA1–CA3 regions as well as granule cells in the dentate gyrus with immunohistochemical staining (Kawato, 2000). Kawato (2000) also showed co-localization of these P450s with StAR in pyramidal neurons and granule cells. Thus, neurons as well as glial cells are now considered to play a major role in neurosteroid formation and metabolism in the brain. In addition to these neurons, P450scc expression has been reported in neurons in the retinal ganglion, sensory neurons in the dorsal root ganglia and motor neurons in the spinal cord of the rat (Guarneri et al., 1994; Compagnone et al., 1995a).

5. Possible actions of neurosteroids produced in the Purkinje neuron

5.1. Acute actions as a novel modulator of neuron-neuron communications

With previous findings as a background, 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 cerebellar Purkinje cells using the rat (Tsutsui et al., 1997a; Tsutsui and Ukena, 1999, 2000). Inhibitory postsynaptic currents (IPSCs) in Purkinje cells were recorded in a

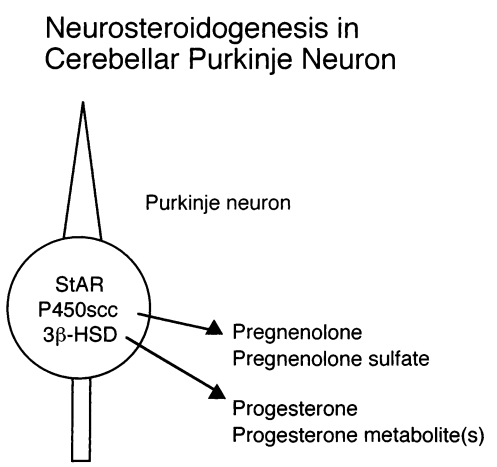

Fig. 10. Neurosteroidogenesis in the cerebellar Purkinje neuron. StAR, P450scc and 3β-HSD are localized in this neuron. The expression of P450scc remains during neonatal development and in adulthood, indicating the constant production of pregnenolone and its sulfate. This neuron also produces highly progesterone and/or its metabolite(s), due to an increase of 3β-HSD activity, only during neonatal life.

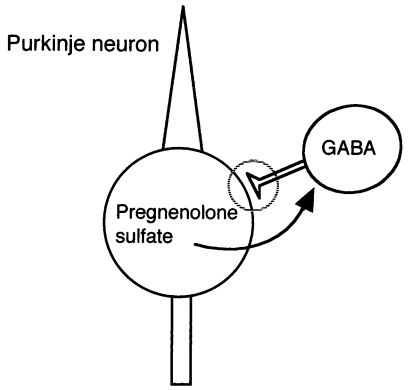

Fig. 11. A model describing the action of pregnenolone sulfate produced in Purkinje neurons. Pregnenolone sulfate may modulate GABAergic transmission by means of the paracrine action on GABAergic neurons.

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. It is therefore possible that pregnenolone sulfate, produced in Purkinje cells, may modulate GABAergic transmission by paracrine actions on GABAergic neurons rather than by genomic mechanisms (Fig. 11).

5.2. Prolonged actions as a classical regulator of gene expressions

Purkinje cells produce not only pregnenolone but also progesterone during the neonatal period, as the expression of 3β-HSD and its enzymatic activity increased in neonatal rats (Figs. 6 and 9) (Ukena et al., 1999a). Progesterone, a sex steroid, is known as an important classical steroid. Progesterone produced in the ovary crosses the blood–brain barrier as a result of its lipid solubility and acts on neurons through intracellular receptor-mediated mechanisms that regulate the transcription of specific genes. Such a genomic action of progesterone contributes to several important brain functions. Therefore, progesterone, produced in Purkinje cells, may also contribute to some important events through its genomic action in the cerebellum during neonatal life (Tsutsui and Ukena, 1999), when drastic morphological changes in the rat cerebellum occur (Altman, 1972a, b). According to Altman (1972a, b), rat Purkinje cells completely differentiate at 3 days of age and locate in a narrow zone between the molecular and granular layers. The external granular layer mainly develops at around 10 days of age, followed by a migration of external granule cells into the granular layer through the Purkinje cells, and the external granular layer disappears. The formation of the cerebellar cortex is almost complete after around 21 days of age. Thus, postnatal development in the cerebellum is dramatic during neonatal life, showing a higher expression of 3β-HSD. More recently, we also found some metabolite(s) of progesterone, such as 3α,5α-tetrahydroxy-progesterone, in the neonatal cerebellum (Tsutsui and Ukena, 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 and glial growth and neuronal synaptic contact (Fig. 10).

6. Other evidence for neurosteroid actions

On the other hand, there is evidence indicating that in mammals, neurosteroids mediate their actions through ion-gated channel receptors, such as $GABA_A$ and *N*-methyl-D-aspartate (NMDA) rather than through intracellular steroid receptors which promote the classical genomic actions (Majewska et al., 1986; Majewska and Schwartz, 1987; Lambert et al., 1990; Lan et al., 1990; Morrow et al., 1990; Puia et al., 1990; Shingai et al., 1991; Wu et al., 1991; Majewska, 1992). For example, pregnenolone and its sulfate ester are thought to act as an agonist and antagonist of GABAergic neurotransmission (Majewska and Schwartz, 1987; Majewska et al., 1988; Mienville and Vicini, 1989). In addition, pregnenolone sulfate potentiates the opening probability of the NMDA subtype of glutamate receptors in cultured neurons (Wu et al., 1991; Bowlby, 1993; Irwin et al., 1992, 1994; Fahey et al., 1995). Progesterone and its metabolite(s) also act through ion-gated channel receptors, such as $GABA_A$ and glycine, to modulate interneuronal communication and excitability as well as through nuclear steroid receptors (Majewska et al., 1986; Lan et al., 1990; Morrow et al., 1990; Puia et al., 1990; Shingai et al., 1991; Majewska, 1992; Paul and Purdy, 1992; Valera et al., 1992; Rupprecht et al., 1993; Patchev et al., 1994). Recently, a physiological function of pregnenolone sulfate with respect to memory has been suggested in rats (Vallée et al., 1997). According to Vallée et al. (1997), hippocampal content of pregnenolone sulfate took part in preserving and/or enhancing cognitive abilities in aged rats, possibly via an interaction with central cholinergic systems. Pregnenolone sulfate, produced in hippocampal pyramidal neurons (Kawato, 2000), may contribute to memory through mechanisms that potentiate the Ca^{2+} conductivity of NMDA receptors (Wu et al., 1991; Bowlby, 1993; Irwin et al., 1992, 1994; Fahey et al., 1995).

It has also been found that progesterone promotes myelination in the peripheral nervous system (Koenig et al., 1995). In addition, 3α,5α-tetrahydroxy-progesterone may regulate nerve growth in rat cultured neurons (Brinton, 1994). These results are in agreement with our hypothesis that progesterone and/or 3α,5α-tetrahydroxy-progesterone may be involved in the formation of the cerebellar neuronal circuit in neonatal life. On the other hand, progesterone and 3α,5α-tetrahydroxy-progesterone may enhance sexual motivation, receptivity and proceptivity through membrane receptor-mediated mechanisms in female rats and mice (Frye and Gardiner, 1996; Frye et al., 1998; Frye and Vongher, 1999).

In addition to mammals, knowledge has been accumulating in non-mammalian species. Pregnenolone sulfate and progesterone may contribute to the display of reproductive behaviors in both wild amphibian and avian species (Askew et al., 1997; Clark et al., 1999; Takase et al., 1999). In this seasonally breeding amphibian, P450scc appeared in not only the cerebellar Purkinje neuron but also the nucleus preopticus and the nucleus infundibularis ventralis in the diencephalon (Takase et al., 1999), areas known to be involved in the control of reproductive behaviors and also contain GABA-like immunoreactive neurons (Franzoni and Morino, 1989; Reichenberger et al., 1993). GABA is accepted as an inhibitory neurotransmitter, and pregnenolone sulfate is generally considered to act as an antagonist of GABAergic neurotransmission (Majewska and Schwartz, 1987; Majewska et al., 1988; Mienville and Vicini, 1989). Accordingly, a higher level of pregnenolone sulfate during the active season may act as a stimulatory modulator to increase neuronal activity in the amphibian diencephalon. It is possible that the amphibian quiescent phase might change to the active phase by such an action of pregnenolone sulfate.

A similar seasonal change in progesterone was evident in the brain of ring doves, a seasonally breeding bird (Clark et al., 1999). Progesterone levels in the male dove diencephalon increased during the brooding season, as a consequence of an increase in the 3β-HSD activity (Clark et al., 1999). In the ring dove, the transition from courtship to parental and associated aggressive behaviors is induced by progesterone. The expression of progesterone receptors was higher in the preoptic region in male doves during the parenting period, whereas plasma progesterone levels were exhibited at a low level throughout the breeding cycle (Askew et al., 1997). Therefore, it is probable that the increase in progesterone in the diencephalon may mediate the transition to and maintenance of parental behavior of the male birds. On the other hand, Moore and his colleagues (Moore and Orchinik, 1994; Rose et al., 1995) suggest that membrane receptors for corticosterone may be involved in a rapid behavioral responses of amphibian courtship behavior, although there has been no demonstration of the biosynthesis of corticosteroids in the vertebrate brain.

7. Conclusions and future directions

De novo steroidogenesis from cholesterol is now established in the vertebrate brain. Steroids synthesized in the brain as well as other nervous system are called neurosteroids. The pioneering discovery of Baulieu and his colleagues with mammals and our studies with non-mammals have opened the door of a new research field. In addition to understand the physiological role of neurosteroids in brain function, it is essential to identify the cells involved in neurosteroidogenesis. Glial cells were first demonstrated as a major site for neurosteroid formation and metabolism in the brain. Subsequently, a series of our studies demonstrated that the Purkinje cell, a cerebellar neuron, possesses the neurosteroidogenic enzymes cytochrome P450scc and 3β-HSD and produces pregnenolone, pregnenolone sulfate and progesterone from cholesterol in mammals as well as non-mammals. This is the first observation for neuronal neurosteroidogenesis in the brain. The expression of StAR, which regulates cholesterol availability in mitochondria, was also demonstrated in the Purkinje neuron. Thus, the cerebellar Purkinje neuron is considered to be a typical site for neurosteroid formation. In addition to the Purkinje neuron, neuronal neurosteroidogenesis was recently found in other mammalian neurons, such as pyramidal neurons in the hippocampus, neurons in the retinal ganglion, sensory neurons in the dorsal root ganglia, etc. Both glial and neuronal cells are now accepted to produce neurosteroids in the vertebrate brain.

In the Purkinje neuron, cytochrome P450scc appears immediately after its differentiation. The expression of P450scc remains during neonatal development and in adulthood, indicating the constant production of pregnenolone and its sulfate. In addition, this neuron produces highly progesterone, as a product of an increase of 3β-HSD activity, only in a limited neonatal period. Pregnenolone sulfate, produced in mammalian Purkinje neurons, may modulate GABAergic transmission by paracrine actions on GABAergic neurons rather than genomic mechanisms. On the other hand, progesterone and/or its metabolite(s) may be involved in the formation of the cerebellar neuronal circuit which occurs in neonatal life.

In the hippocampus, pregnenolone sulfate, produced in pyramidal neurons, may also act as a neuromodulator on neurons to influence interneuronal communications due to the potentiation of Ca^{2+} conductivity of NMDA receptors. Interestingly, both cerebellar Purkinje neurons and hippocampal pyramidal neurons are

involved in memory and learning. Thus, the discovery of neurosteroidogenesis in these neurons serves as an excellent model for future study of physiological roles of neurosteroids in the brain. Neurosteroids produced in these neurons may play an essential role in the process of memory and learning. The hypothesis postulated here is partly confirmed by recent findings (Flood et al., 1992; Vallée et al., 1997). For example, hippocampal content of pregnenolone sulfate preserved and/or enhanced cognitive abilities in aged animals, possibly via an interaction with central cholinergic systems (Vallée et al., 1997). Therefore, future attention should be focused on behavioral studies using neurosteroidogenic enzyme-knockout animals and electrophysiological studies on the occurrence of long-term potentiation (LTP) and/or long-term depression (LTD).

Neurosteroids are synthesized by neurons as well as glial cells. Contrary to the endocrine actions of peripheral steroid hormones, they have paracrine/autocrine actions in the brain. Available data suggest that (1) neurosteroids have effects on memory and learning by modulating the function of neurotransmitter receptors; (2) neurosteroids have also effects on other brain functions, such as instinctive and emotional processes, by a similar mechanism; and (3) neurosteroids promote neuronal and glial growth and neuronal synaptic contact by regulating gene expression. Thus, this new concept has important biomedical implications.

Up to now the regulatory mechanisms, which govern the biosynthesis of neurosteroids in neurons, are unknown. Seasonal changes in neurosteroids in wild animals may be associated with changes in some environmental cues. Further study in neurons is required to identify environmental cues and brain factors that regulate neurosteroidogenesis.

Acknowledgements

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