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**[REVIEW]**

# Biosynthesis and Biological Actions of Neurosteroids in Brain Neurons

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## 1. INTRODUCTION TO NEUROSTEROID RESEARCH

### *(1) Classical concept: Brain is a target site of peripheral steroids*

Steroid hormones supplied by the peripheral steroidogenic glands regulate several important brain 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). By diverse actions on the brain, peripheral steroids, in particular sex steroids, have profound effects on behavior of vertebrate animals. Extensive studies have been conducted to understand the mechanisms for steroid actions on several kinds of behaviors including courtship, copulatory, aggressive and parental behaviors. Some approach has been to measure sex steroid levels in blood, and to correlate these hormone levels with the display of discrete behaviors. These kinds of studies on a variety of wild and captive, intact and castrated, reproductive and non-reproductive animals have established a relationship between the presence and activation of the gonads, increased blood levels of androgens, estrogens or progestins in adult males and females, and expression of adult typical reproductive behaviors.

Gonadal androgens, for instance, act on the brain to influence several male reproductive behaviors in vertebrates. Castration of adult male birds leads to decreases or losses of aggressive, courtship, and copulatory behaviors and replacement therapy with androgens restores these behaviors (Adkins and Adler, 1972; Arnold, 1975; Pröve, 1978, Tsutsui and Ishii, 1981; Ishii and Tsutsui, 1982; Balthazart, 1983; Wingfield and Marler, 1988; Wingfield and Farner, 1993). Many of the brain regions that control a variety of reproductive behaviors con-

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tain a high proportion of cells that concentrate androgenic hormones in male birds (Arnold *et al.*, 1976; Korsia and Bottjer, 1989; Watson and Adkins-Regan, 1989). Therefore, the brain is considered to be a target site of peripheral steroids.

***(2) New evidence: Brain is also a steroidogenic site***

As mentioned above, a great deal was known about the brain as a target site of steroid hormones more than 10 years before. On the other hand, new findings from several laboratories over the past decade have established unequivocally that the nervous system itself forms steroids *de novo* from cholesterol. The pioneering discovery of Baulieu and his colleagues using mammals (for a review, see Baulieu, 1997) and our studies with nonmammals (for a review, see Tsutsui *et al.*, 1999) have opened the door of a new research field. The new concept that steroids could be synthesized *de novo* in the brain derived from observations made by Baulieu and colleagues. They found that several steroids such as pregnenolone, dehydroepiandrosterone, and their sulfate and lipoidal esters highly accumulated within the brain 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 remained constant even after the removal of peripheral steroids by procedures such as adrenalectomy, castration and hypophysectomy. These results suggested that the brain can synthesize steroids *de novo* from cholesterol (Corpéchot *et al.*, 1981, 1983; Robel and Baulieu, 1985; Robel *et al.*, 1986, 1987; Jo *et al.*, 1989). In contrast to mammalian studies, little has been known regarding *de novo* steroidogenesis in the brain of nonmammalian vertebrates. We therefore looked for steroids formed from cholesterol in the brain of birds (Tsutsui and Yamazaki, 1995; Usui *et al.*, 1995; Tsutsui *et al.*, 1997a, 1997b; Ukena *et al.*, 1999b, 2001; Matsunaga *et al.*, 2001; Tsutsui and Schlinger, 2001), amphibians (Takase *et al.*, 1999) and fish (Sakamoto *et al.*, 2001a). 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 both mammalian and nonmammalian vertebrates. Such steroids synthesized in vertebrate brains are called neurosteroids.

***(3) A model for the study of biosynthesis and biological actions of neurosteroids***

When we understand the physiological role of neurosteroids in brain functions, it is essential to identify the cells involved in neurosteroidogenesis. In recent years knowledge has been accumulated in both mammals and nonmammals that glial cells play an important 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 brain neurons. Interestingly, the Purkinje cell, a typical cerebellar neuron, possesses neurosteroidogenic enzymes and produces neurosteroids *de novo* in a variety of vertebrates including mammalian species (Usui *et al.*, 1995; Tsutsui *et al.*, 1997a, 1997b; Ukena *et al.*, 1998; Takase *et al.*, 1999; Ukena *et al.*, 1999a). This is the first discovery of neuronal *de novo* neurosteroidogenesis in the brain and serves as an excellent model for the study of neurosteroid actions in the brain.

This review summarizes the advances made in our understanding of biosynthesis and biological actions of neurosteroids in neurons. For detailed information of neurosteroids in glial cells the reader is referred to excellent reviews (Baulieu, 1997; Compagnone and Mellon, 2000).

## 2. NEUROSTEROIDS IN VERTEBRATE BRAINS

***(1) Biosynthesis of neurosteroids in mammalian brains***

Pregnenolone, a 3β-hydroxy-$\Delta^5$-steroid, is a main precursor of steroid hormones secreted by peripheral steroidogenic glands, such as gonads, adrenals, and placentae. 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 glandular cells. Therefore, it is essential to demonstrate the formation of pregnenolone in the brain. A number of studies with mammals have reported that the brain contains abundant quantities of 3β-hydroxy-$\Delta^5$-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 shown 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, one of the first neurosteroids identified in the adult rat 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*-$\Delta^4$-steroid, and its metabolites, such as 3α-dihydroprogesterone and 3α,5α-tetrahydroprogesterone, are also produced and accumulate in the mammalian brain as neurosteroids. The biosynthesis of

progesterone is performed by 3β-hydroxysteroid dehydrogenase/$\Delta^5$-$\Delta^4$-isomerase (3β-HSD) which catalyzes the dehydrogenation and isomerization of the $\Delta^5$-3β-hydroxysteroids (pregnenolone and dehydroepiandrosterone) into $\Delta^4$-ketosteroids (progesterone and androstenedione, respectively) and is highly expressed in the peripheral steroidogenic glands (Mason, 1993). The expression of both 3β-HSD protein and its mRNA has been reported in mammalian brains (Dupont *et al.*, 1994; Guennoun *et al.*, 1995a; 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) Biosynthesis of neurosteroids in nonmammalian brains*

The concept of *de novo* steroidogenesis from cholesterol in the brain of nonmammalian vertebrates derived from our observations made in 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 quail brain using a specific radioimmunoassay. The pregnenolone concentration in adult birds was much higher in the brain than in plasma. The accumulation of pregnenolone in the quail brain may be largely independent of peripheral steroidogenic glands, because a high level of pregnenolone persisted in the hypophysectomized birds. Pregnenolone sulfate ester was also detectable in the avian brain. Subsequently, the formation of pregnenolone from cholesterol was found in intact mitochondria derived from the quail brain (Tsutsui and Yamazaki, 1995). 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 the brain, 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 brain of another bird, the ring dove (Clark *et al.*, 1999; Tsutsui *et al.*, 1999; Lea *et al.*, 2001). Taken together, these biochemical and immunochemical studies indicate that avian brains possess cytochrome P450scc and produce pregnenolone from cholesterol (for reviews, see Tsutsui *et al.*, 1997a, 1997b, 1999).

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. The concentrations of pregnenolone and its sulfate ester in the brain of *Xenopus laevis* were greater than those in the gonads and plasma (Takase *et al.*, 1999). An immunoreactive protein band of electrophoretic mobility in the proximity of bovine P450scc was detected in the *Xenopus* brain by Western blot analysis (Takase *et al.*, 1999). As a lower vertebrate species also possesses P450scc in the brain, the presence of P450scc is considered as a conserved property of vertebrate brains. 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. Ukena and Tsutsui (2001) further demonstrated that the embryonic avian brain actively metabolizes progesterone to 5β-dihydroprogesterone. In addition to these nonmammalian vertebrates, the expression of 3β-HSD and progesterone formation were also obtained in the brain of zebrafish (Sakamoto *et al.*, 2001a). Thus, it is now established that *de novo* steroidogenesis from cholesterol occurs in vertebrate brains (Tsutsui *et al.*, 1999).

### *(3) Biosynthetic pathway of neurosteroids*

In the peripheral steroidogenic glands, the production of steroid hormones requires the coordinate action of steroidogenic enzymes that start with cholesterol as the initial substrate, and catalyze a series of reactions that ultimately produce several kinds of steroids. If a variety of neurosteroids are synthesized in the brain, then each of these enzymes must be present in the vertebrate brain. 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-$\Delta^5$-steroids and 3-oxo-$\Delta^4$-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 (Kohchi *et al.*, 1998). On the other hand, 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 during neonatal life, 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, such as progesterone and its metabolites, of 3β-HSD activity.

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.* (1995a) reported that rat embryonic cells expressing $P450_{17\alpha,lyase}$ are located in the mesencephalic region as well as the medulla and spinal cord. Our recent studies also indicated the expression of $P450_{17\alpha,lyase}$ mRNA in the quail brain (Matsunaga *et al.*, 2001). This enzyme was also highly expressed in the quail mesencephalon (Matsunaga *et al.*, 2001). 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. Further study is needed to obtain the detailed understanding of neurosteroid production in specific brain regions and different developmental stages.

## 3. PHYSIOLOGICAL CHANGES IN NEUROSTEROIDS IN THE VERTEBRATE BRAIN

*De novo* steroidogenesis from cholesterol appears in the brain of several vertebrates, as described above. Physiological changes in neurosteroid concentrations in the brain must be taken into account when understanding the function of neurosteroids in the vertebrate brain. If neurosteroids are involved in important brain functions, we expect 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 with a short breeding period. Such a variation is the consequence of interaction between external environmental and internal factors. 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). 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 brain pregnenolone concentrations were virtually constant throughout the year (Takase *et al.*, 1999). 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 (Takase *et al.*, 1999). We further found a seasonal change in progesterone in the brain of newt *Cynops pyrrhogaster*. The progesterone concentration in this wild urodele brain was maximal in the breeding season in both sexes (Inai *et al.*, unpublished). A seasonal change in progesterone in the urodele brain was also independent of the plasma steroid level (Inai *et al.*, unpublished).

Recently, we have collaborated with Lea and his colleagues to analyze seasonal changes in neurosteroid concentrations using a seasonally breeding bird, the ring dove *Streptopelia risoria*. This bird also showed a seasonal change in progesterone in the brain (Clark *et al.*, 1999; Tsutsui *et al.*, 1999). Progesterone concentrations in the male dove diencephalon may increase during the brooding season, as a consequence of an increase in the 3β-HSD activity ( Lea *et al.*, unpublished). It is considered that 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 area in male doves during the parenting period, whereas plasma progesterone concentrations were low throughout the breeding cycle (Askew *et al.*, 1997). Accordingly, the increase of progesterone produced in the diencephalon may mediate the transition to and maintenance of parental behavior of the male birds.

## 4. NEUROSTEROIDOGENESIS IN BRAIN NEURONS

### *(1) Identification of neurosteroidogenic cells*

Identification of neurosteroidogenic cells in the brain is essential to analyze the action of neurosteroids. 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 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). This is also true for the presence of P450scc in glial cells located in the telencephalic and diencephalic regions of the quail (Usui *et al.*, 1995; Tsutsui *et al.*, 1997a) and the ring dove (Lea *et al.*, 2001).

In contrast to glial cells, the concept of *de novo* neurosteroidogenesis in neurons in the brain has been uncertain. We have found the cerebellar neuron to be an active neurosteroidogenic cell, which possesses both cytochrome P450scc and 3β-HSD and produces pregnenolone, pregnenolone sulfate and progesterone, in several vertebrate species (Fig. 1) (Usui *et al.*, 1995; Tsutsui *et al.*, 1997a, 1997b; Ukena *et al.*, 1998; Takase *et al.*, 1999; Ukena *et al.*, 1999a).

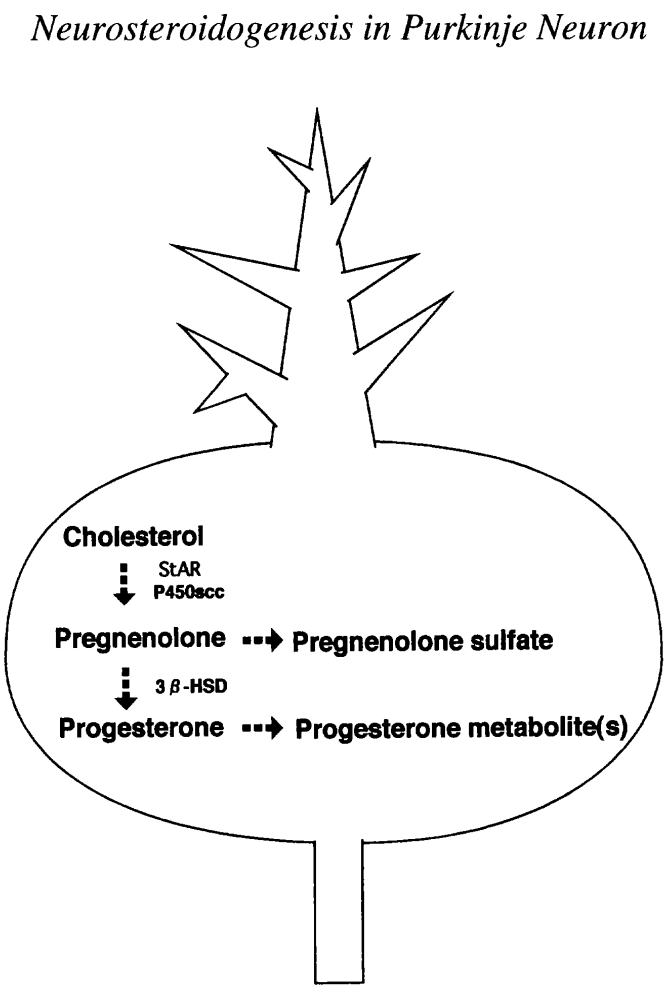


**Fig. 1. Biosynthetic pathway for neurosteroids in the cerebellar Purkinje neuron.**
StAR, P450scc and 3β-HSD are localized in this neuron. The expression of P450scc remains during neonatal development and in adulthood, indicating the constant production of pregnenolone and its sulfate. This neuron also produces highly progesterone and/or its metabolite(s), due to an increase of 3β-HSD activity, only during neonatal life. StAR: steroidogenic acute regulatory protein, P450scc: cytochrome P450 side-chain cleavage enzyme, 3β-HSD: 3β-hydroxysteroid dehydrogenase/$\Delta^5$-$\Delta^4$-isomerase

Thus, our studies provided the first evidence for the location of P450scc and 3β-HSD in the brain neuron and gave the opportunity to understand neuronal neurosteroidogenesis in the brain.

***(2) Purkinje neuron is a major site of neurosteroidogenesis***

In our immunohistochemical studies of the quail brain using an antibody against P450scc, the 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, 1997b). 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, 1997b). Western immunoblot analysis confirmed the presence of P450scc in Purkinje cells (Usui *et al.*, 1995). These findings obtained in the avian brain have provided the first evidence for the location of cytochrome P450scc in neurons in the brain, because the Purkinje cell is a typical cerebellar neuron.

Whether neurons located in the brain of other vertebrate species possess cytochrome P450scc and produce pregnenolone and its sulfate ester still remained unclear. Therefore, we investigated the presence of P450scc in the cerebellar Purkinje neuron using a mammalian species (Ukena *et al.*, 1998). Immunoreaction with P450scc was confined to the somata and dendrites of Purkinje neurons in the rat cerebellum (Ukena *et al.*, 1998). An antibody against inositol triphosphate ($IP_3$) receptor, a marker of the Purkinje neuron, recognized P450scc-immunoreactive cerebellar cells that showed no immunoreaction with glial fibrillary acidic protein (GFAP), a specific marker of glial cells (Ukena *et al.*, 1998). In addition, the expressions of both P450scc protein and P450scc mRNA were detected in the rat cerebellum (Ukena *et al.*, 1998). Interestingly, P450scc appeared in the rat Purkinje neuron immediately after its differentiation and the expression of this enzyme persisted during neonatal development into adulthood (Ukena *et al.*, 1998). In addition to higher vertebrates, our recent studies with amphibians further identified the presence of P450scc in the cerebellar Purkinje neuron of *Xenopus laevis* and *Rana nigromaculata* (Takase *et al.*, 1999). Taken together, these findings obtained in both higher and lower vertebrates (Usui *et al.*, 1995; Tsutsui *et al.*, 1997a, 1997b; Ukena *et al.*, 1998; Takase *et al.*, 1999) indicate that Purkinje neurons possess P450scc and produce pregnenolone and its sulfate ester (Fig. 1).

Subsequently, we have extended our understanding of 3β-HSD expression in this neuron. RT-PCR and biochemical analyses showed the expression of 3β-HSD and its enzymatic activity in the cerebellum of neonatal and adult rats (Ukena *et al.*, 1999a). Employing *in situ* hybridization of 3β-HSD mRNA, the site of 3β-HSD expression was localized in Purkinje neurons and external granule cells (Ukena *et al.*, 1999a). Thus, both P450scc and 3β-HSD are expressed in Purkinje neurons (Fig. 1). The expression of 3β-HSD, however, increased during the neonatal period, unlike P450scc (Ukena *et al.*, 1999a). In contrast to a constant production of pregnenolone, Purkinje neurons produced not only progesterone but also its metabolite(s) during neonatal life (Fig. 1) (Tsutsui and Ukena, 2000; Tsutsui *et al.*, 2001). Such an age-dependent expression of 3β-HSD was confirmed by biochemical studies together with HPLC analysis, indicating an increase of progesterone formation during neonatal life (Ukena *et al.*, 1999a). Notwithstanding such a difference in the age-dependent expression, our studies have demonstrated that the Purkinje neuron is an important neurosteroidogenic cell in the vertebrate brain (Fig. 1) (for reviews, see Tsutsui and Ukena, 1999; Tsutsui *et al.*, 1999, 2000, 2001).

On the other hand, the steroidogenic acute regulatory protein (StAR) has recently been found in Purkinje neurons (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 bio-

synthesis in the peripheral steroidogenic glands (Clark *et al.*, 1994; Stocco and Clark, 1996). StAR may also contribute to the regulation of neurosteroidogenesis in the Purkinje neuron (Fig. 1).

***(3) Other neurons***

Recently, the localization of nurosteroidogenic enzymes in other brain neurons has been characterized. For instance, the expressions of P450scc, $P450_{17\alpha,lyase}$, and P450arom were detected in the rat hippocampal neurons (Kawato *et al.*, 1999). In addition to brain neurons, P450scc was also found in neurons of 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*, 1995b).

## 5. BIOLOGICAL ACTIONS OF NEUROSTEROIDS PRODUCED IN PURKINJE NEURONS

***(1) Purkinje neuron serves as an excellent model for the study of neurosteroid actions***

To understand neurosteroid actions in the brain, we need data on the specific synthesis in particular sites of the brain at particular times. Such informations are crucial to allow one to develop hypotheses predicting the potential roles of particular neurosteroids in the developing or adult brain. Therefore, studies for this exciting area of research should be focused on the mode of action of neurosteroids produced locally in the identified neurosteroidogenic cells underlying important brain functions. We have identified the Purkinje neuron as a major site of neurosteroidogenesis in the brain. This neuron expresses several kinds of neurosteroidogenic enzymes in a variety of vertebrates (for reviews, see Tsutsui and Ukena, 1999; Tsutsui *et al.*, 1999, 2000, 2001). In addition, the Purkinje neuron is known to play an important role in the process of memory and learning. Thus, this neuron may serve as an excellent cellular model for the study of neurosteroid actions.

***(2) Nongenomic actions as a novel neuromodulator of neurotransmission***

Until recently, we believed that all steroid hormones regulate biological functions by genomic mechanisms. The genomic action of steroid hormones presumes that steroid hormones cross the plasma membrane and bind to and activate specific intracellular steroid receptors. The activated steroid receptors modulate gene transcription and protein synthesis. However, new findings have been obtained that some neurosteroids, such as pregnenolone, pregnenolone sulfate, progesterone and progesterone metabolite(s), may mediate their actions through ion-gated channel receptors rather than by genomic mechanisms. Our recent studies have focused on neurosteroid actions in the Purkinje neuron and indicated that pregnenolone sulfate contributes to important events in the cerebellum by nongenomic mechanisms.

To understand the mode of action of neurosteroids, produced in Purkinje neurons, we examined the effects of pregnenolone and its sulfate ester on synaptic currents in Purkinje

*Nongenomic Action of Pregnenolone Sulfate*

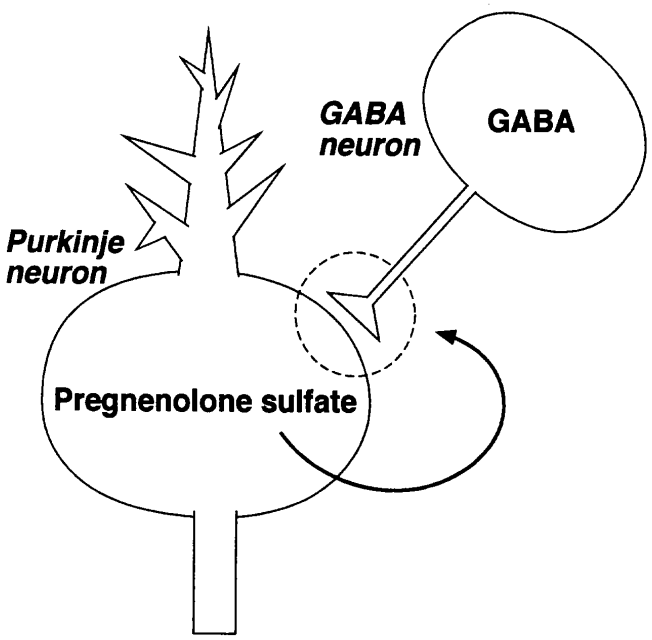


**Fig. 2. Nongenomic action of pregnenolone sulfate produced in Purkinje neurons.**
Pregnenolone sulfate modulates GABAergic transmission by means of the nongenomic action on GABAergic neurons.

neurons using the rat (Tsutsui *et al.*, 1997a; Tsutsui and Ukena, 1999; Tsutsui and Ukena, 2000; Tsutsui *et al.*, 2000). Inhibitory postsynaptic currents (IPSCs) in Purkinje neurons were recorded in a cerebellar slice by the patch-clamp method. Pregnenolone sulfate increased, in a dose-related way, the frequency of IPSCs within 1 min of perfusion, indicating that this effect is unlikely to be induced *via* gene transcription. In contrast, pregnenolone had no effect on the frequency of IPSCs. The IPSCs recorded in the Purkinje neurons were completely blocked by bicuculline, a γ-aminobutyric acid A ($GABA_A$) receptor antagonist, suggesting that they are mediated by $GABA_A$ receptors. Thus, pregnenolone sulfate, produced in Purkinje neurons, may modulate GABAergic transmission by nongenomic actions on GABAergic neurons rather than by genomic mechanisms (Fig. 2).

***(3) Genomic actions on neuronal growth and synaptogenesis***

Purkinje neurons 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 (Ukena *et al.*, 1999a). Recently, we also found some metabolite(s) of progesterone, such as 3α,5α-tetrahydroprogesterone, in the neonatal cerebellum (Tsutsui and Ukena, 2000; Tsutsui *et al.*, 2001). It is well known that in the rat cerebellum dramatic morphological changes occur during neonatal life. According to Altman (1972a, 1972b), rat Purkinje neurons 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 neurons, 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 in the Purkinje neuron. 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, produced as a neurosteroid in the Purkinje neuron only during neonatal life, on neuronal growth in the cerebellum. Interestingly, *in vitro* studies using cultured cerebellar slices of newborn rats showed that progesterone promotes dendritic growth of the Purkinje neuron (Sakamoto *et al.*, 2001b). A similar result was obtained by *in vivo* studies. Electron microscopic analysis further revealed that progesterone induces an increase of the density of synapses on the Purkinje neuron (Sakamoto *et al.*, 2001b). Furthermore, intranuclear receptors for progesterone were expressed in the Purkinje neuron (Sakamoto *et al.*, 2001b). In contrast to progesterone, we could not detect any significant effects of 3α,5α-tetrahydroprogesterone, a progesterone metabolite, on Purkinje development. These results indicate that progesterone promotes the dendritic growth and synaptogenesis of Purkinje neurons by genomic mechanisms (Fig. 3) (Sakamoto *et al.*, 2001b; Tsutsui *et al.*, 2001). Such an action of progesterone may contribute to the formation of the cerebellar neuronal circuit during neonatal life.

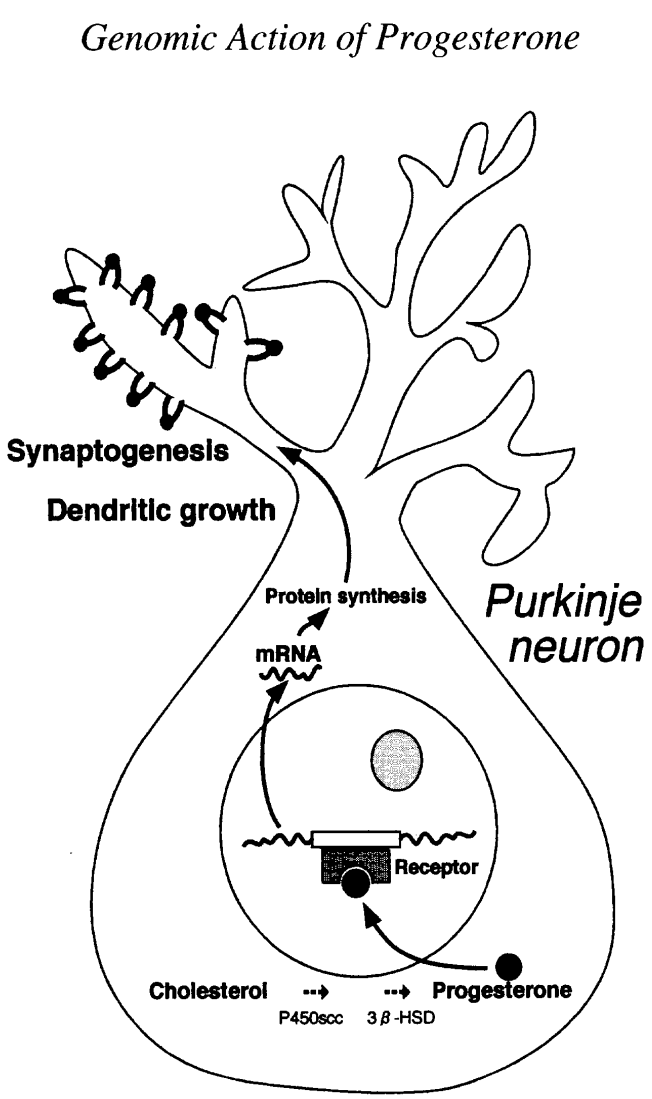


**Fig. 3. Genomic action of progesterone produced in Purkinje neurons.**
Progesterone acts on the Purkinje neuron through intracellular receptor-mediated mechanisms that promote dendritic growth and synaptogenesis in this neuron by genomic mechanisms. Such an action of progesterone may contribute to the formation of the cerebellar neuronal circuit during neonatal life.

## 6. OTHER EVIDENCE FOR NEUROSTEROID ACTIONS

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 *et al.*, 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 may contribute to memory through mechanisms that potentiate the $Ca^{2+}$ conductivity of NMDA receptors (Wu *et al.*, 1991; Bowlby *et al.*, 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). This result is in agreement with our hypothesis that progesterone may be involved in the formation of the cerebellar neuronal circuit in neonatal life. In addition, 3α,5α-tetrahydroprogesterone may regulate nerve growth in rat cultured neurons (Brinton, 1994). On the other hand, progesterone and 3α,5α-tetrahydroprogesterone 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).

Dehydroepiandrosterone and its sulfate ester are also abundant neurosteroids in the brain (Corpéchot *et al.*, 1981, 1983; Jo *et al.*, 1989). Recently, Compagnone and Mellon (1998) reported a stimulatory action of these neurosteroids on neuronal growth using primary cultures of mouse embryonic neocortical neurons. Dehydroepiandrosterone selectively increased the length of axons and the incidence of varicosities and basket-like process formations *in vitro*, whereas dehydroepiandrosterone sulfate selectively promoted branching and dendritic growth *in vitro* (Compagnone and Mellon, 1998). We also reported a stimulatory action of progesterone on dendritic growth and synaptogenesis in Purkinje neurons during cerebellar cortical formation (Sakamoto *et al.*, 2001b). Therefore, these neurosteroids may play an important role in cortical organization in both the cerebellum and cerebrum during development.

## 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. Neurosteroidogenic enzymes are expressed in both neurons and glial cells. The Purkinje cell, a cerebellar neuron, is considered to play a major role in neurosteroid formation and metabolism in the brain. This neuron possesses the neurosteroidogenic enzymes cytochrome P450scc and 3β-HSD and produces pregnenolone, pregnenolone sulfate and progesterone from cholesterol. Cytochrome P450scc appears in the Purkinje neuron immediately after its differentiation. The expression of P450scc remains during neonatal development and in adulthood, indicating the constant production of pregnenolone and its sulfate. This neuron also produces significant amounts of progesterone, as a product of an increase of 3β-HSD activity, only in a limited neonatal period. Pregnenolone sulfate modulates GABAergic transmission by nongenomic actions on GABAergic neurons rather than genomic mechanisms. On the other hand, progesterone is involved in the promotion of dendritic growth and synaptogenesis of the Purkinje neuron by genomic mechanisms. This action of progesterone may contribute to the formation of the cerebellar neuronal circuit during neonatal life. These serve as an excellent model for the study of physiological roles of neurosteroids in the cerebellum, because Purkinje neurons play an important role in the process of memory and learning. 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). In other brain regions, future study is also required to clarify the mode of action of neurosteroids produced locally in the identified cells underlying important brain functions.

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