Biosynthesis and Biological Actions of Pineal Neurosteroids in Domestic Birds

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Key Words

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Abstract

The central and peripheral nervous systems have the capacity of synthesizing steroids *de novo* from cholesterol, the so-called 'neurosteroids'. *De novo* synthesis of neurosteroids from cholesterol appears to be a conserved property across the subphylum vertebrata. Until recently, it was generally believed that neurosteroids are produced in neurons and glial cells in the central and peripheral nervous systems. However, our recent studies on birds have demonstrated that the pineal gland, an endocrine organ located close to the brain, is an important site of production of neurosteroids *de novo* from cholesterol. 7 α -Hydroxypregnenolone is a major pineal neurosteroid that stimulates locomotor activity of juvenile birds, connecting light-induced gene expression with locomotion. The other major pineal neurosteroid allopregnanolone is involved in Purkinje cell survival by suppressing the activity of caspase-3, a crucial mediator of apoptosis during cerebellar development. This review is an updated summary of the biosynthesis and biological actions of pineal neurosteroids.

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The brain has traditionally been considered as a target site for peripheral steroid hormones in vertebrates. However, studies conducted over the past two decades have demonstrated that the central and peripheral nervous systems have the capacity of synthesizing steroids, the so-called 'neurosteroids' [for reviews, see 1–9]. *De novo* formation of neurosteroids in the brain was originally demonstrated in mammals [10–18], and subsequently in birds [2, 6, 19–35], amphibians [36–47] and fish [48–51]. Therefore, *de novo* synthesis of neurosteroids from cholesterol in the brain appears to be conserved across the subphylum vertebrata [1–9].

Until recently, it was generally accepted that neurosteroids are produced in neurons and glial cells in the central and peripheral nervous systems [for reviews, see 1–9]. However, there is now new evidence that, in the juvenile birds, the pineal gland, an endocrine organ located close to the brain, actively produces a variety of neurosteroids *de novo* from cholesterol [52, 53]. Notably, 7 α -hydroxypregnenolone and allopregnanolone (3 α ,5 α -tetrahydroprogesterone; 3 α ,5 α -THP) are major neurosteroids secreted by the pineal gland [52, 53]. Importantly, the pineal gland produces 7 α -hydroxypregnenolone stimulating locomotor activity in light- and circadian time-dependent manners [52]. On the other hand, allopregnanolone produced in the pineal gland prevents cell

death of Purkinje cells by suppressing the activity of caspase-3 in the cerebellum during development [53]. Based on these new findings, this review highlights the biosynthesis and biological actions of pineal neurosteroids.

Studies on Neurosteroidogenesis in Birds

Birds have served as excellent animal models for the investigation of neurosteroidogenesis in the brain. It has been demonstrated that the brain of the Japanese quail possesses cytochrome P450 side-chain cleavage enzyme (P450_{scc}; gene name *Cyp11a*), 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase (3 β -HSD; gene name *Hsd3b*), 5 β -reductase (gene name *Srd5b*), cytochrome P450 17 α -hydroxylase/c17,20-lyase (P450_{17 α ,lyase}; gene name *Cyp17*), 17 β -hydroxysteroid dehydrogenase (17 β -HSD; gene name *Hsd17b*), etc., and produces pregnenolone, progesterone, epipregnanolone (3 β ,5 β -tetrahydroprogesterone; 3 β ,5 β -THP), androstenedione, testosterone, and estradiol-17 β from cholesterol [2, 6, 19–25]. The expression and activity of cytochrome P450 aromatase (P450_{arom}; gene name *Cyp19*), which converts testosterone into estradiol-17 β , have also been demonstrated in the quail brain [54–65]. Independently, neurosteroidogenesis in the brain has been demonstrated in the zebra finch [27–35]. The formation and metabolism of neurosteroids from cholesterol is now known to occur in the brain of various birds. Currently, studies on avian neurosteroids are an area of great interest to many researchers.

Recently, 7 α -hydroxypregnenolone has been identified as a novel bioactive neurosteroid in the brain of the Japanese quail [66]. Importantly, 7 α -hydroxypregnenolone acts on brain tissue as a novel neuronal modulator to stimulate locomotor activity of quail [66]. The presence of 7 α -hydroxypregnenolone and a stimulatory action on locomotion were also demonstrated in the brain of the Japanese red-bellied newt [40]. Subsequently, it was found that cytochrome P450 7 α -hydroxylase (cytochrome P450_{7 α} ; gene name *Cyp7b*) catalyzes the conversion of pregnenolone to 7 α -hydroxypregnenolone in the brain of these species [66, 67]. It was further demonstrated that melatonin acts on cytochrome P450_{7 α} -expressing neurons to down-regulate 7 α -hydroxypregnenolone synthesis, thus inducing diurnal locomotor changes in quail [66].

It is known that in birds and other vertebrates, the locomotor activity undergoes a circadian change [68] controlled by the diurnal rhythm of melatonin secretion [69–74]. The discovery of the role of 7 α -hydroxypregnenolone

in mediating the action of melatonin on diurnal locomotor rhythmicity is an important step in understanding the molecular mechanism underlying melatonin regulation of the behavior [66]. A similar mechanism may be evident in other vertebrates [for reviews, see 75–79], because 7 α -hydroxypregnenolone is also produced in the brain of newts [40] and mammals [80–83].

Biosynthesis of Neurosteroids in the Pineal Gland

Neurosteroidogenesis in the Pineal Gland

It was generally believed that neurosteroids are produced only in the central and peripheral nervous systems. However, recent studies carried out in chickens [52] and quail [53] have demonstrated that the pineal gland, an endocrine organ located close to the brain, actively synthesizes neurosteroids *de novo* from cholesterol *via* pregnenolone (fig. 1).

Pregnenolone is known to be the common precursor of all steroid hormones in vertebrates. The formation of pregnenolone is initiated by cleavage of the cholesterol side-chain by cytochrome P450_{scc}, a rate-limiting mitochondrial enzyme originally found in peripheral steroidogenic glandular cells. Therefore, the demonstration of pregnenolone formation from cholesterol is essential to establish *de novo* neurosteroidogenesis in the pineal gland. As an initial step in the demonstration of pregnenolone biosynthesis in the pineal gland, we found that cytochrome P450_{scc} is expressed in the pineal gland of juvenile chickens [52] and juvenile quail [53] by reverse transcription polymerase chain reaction (RT-PCR) analysis (fig. 1). Immunohistochemical labeling with cytochrome P450_{scc} antibodies showed intense staining in cells forming follicular structures in the quail pineal gland [53]. Incubation of the pineal glands from quail chicks with tritiated cholesterol led to the formation of a radioactive metabolite that, upon high-performance liquid chromatography (HPLC) analysis, exhibited the same retention time as pregnenolone [53]. The occurrence of pregnenolone in the pineal gland was also demonstrated by gas chromatography-mass spectrometry (GC-MS) analysis [53].

Subsequently, the expressions of several key steroidogenic enzymes, including cytochrome P450_{7 α} , 3 β -HSD, 5 α -reductase, 5 β -reductase, cytochrome P450_{17 α ,lyase}, 17 β -HSD and cytochrome P450_{arom} have been demonstrated in the pineal gland of both juvenile chickens and juvenile quail [52, 53] by RT-PCR analyses (fig. 1). To clarify the biosynthetic pathways of neuros-

Fig. 1. Biosynthetic pathways for neurosteroids in the pineal gland. The arrows indicate the biosynthetic pathways of neurosteroids identified in the pineal glands of juvenile quail. The pineal gland actively produces a variety of neurosteroids *de novo* from cholesterol. 7 α -Hydroxypregnenolone and allopregnanolone are major products secreted by the pineal gland. P450scc, cytochrome

P450 side-chain cleavage enzyme; P450_{7 α} , cytochrome P450 7 α -hydroxylase; 3 β -HSD, 3 β -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase; 3 α -HSD, 3 α -hydroxysteroid dehydrogenase/ Δ^5 - Δ^4 -isomerase; P450_{17 α ,lyase}, cytochrome P450 17 α -hydroxylase/c17,20-lyase; 17 β -HSD, 17 β -hydroxysteroid dehydrogenase; P450arom, cytochrome P450 aromatase. For details, see text.

teroids in the pineal gland, biochemical studies combined with HPLC and GC-MS analyses were further conducted. Pineal gland homogenates from quail chicks were incubated with tritiated pregnenolone, and subsequent analysis of the products by reversed-phase HPLC detected the formation of 7 α - and/or 7 β -hydroxypregnenolone [53] (fig. 1). In addition, progesterone, allopregnanolone and/or epipregnanolone, androstenedione, testosterone, 5 α - and/or 5 β -dihydrotestosterone and estradiol-17 β were produced from the precursor pregnenolone [53] (fig. 1). Although isomers, such as 7 α - and 7 β -hydroxypregnenolone; allopregnanolone and epipregnanolone; and 5 α - and 5 β -dihydrotestosterone, were not separated from each other by HPLC analysis, the formation of these neurosteroids in the pineal gland was demonstrated by GC-MS analysis [53]. Derivatives of synthetic 7 α - and 7 β -hydroxypregnenolone, progesterone, allopregnanolone, epipregnanolone, androstenedione, testosterone, 5 α - and 5 β -dihydrotestosterone, estradiol-17 β , and the purified nonradioactive steroids produced by the pineal gland were applied to GC-selected ion monitoring (SIM) analysis, which showed the same mass spectral characteristics: m/z 386 for 7 α - and 7 β -hydroxypregnenolone, m/z 510 for progesterone, m/z 514 for allopregnanolone and epipregnanolone, m/z 482 for androstenedione, m/z 680 for testosterone, m/z 486 for 5 α - and 5 β -dihydrotestosterone, and m/z 664 for estradiol-17 β [53]. Unlike HPLC

analysis, GC-MS analysis was capable of separating several pairs of isomers: 7 α - and 7 β -hydroxypregnenolone; allopregnanolone and epipregnanolone; and 5 α - and 5 β -dihydrotestosterone [53]. As summarized in figure 1, the neurosteroids produced in the pineal gland were identified as 7 α - and 7 β -hydroxypregnenolone, progesterone, allopregnanolone, epipregnanolone, androstenedione, testosterone, 5 α - and 5 β -dihydrotestosterone, and estradiol-17 β [53].

In this way, a series of experiments using molecular and biochemical techniques have demonstrated that the pineal gland produces a variety of neurosteroids from cholesterol *via* pregnenolone in the juvenile birds. This is the first observation of *de novo* neurosteroidogenesis in the pineal gland in any vertebrate class.

Major Pineal Neurosteroids

To quantify major neurosteroids synthesized and released in the pineal gland, the pineal glands from quail chicks were cultured in medium 199 with tritiated pregnenolone. HPLC analysis revealed that pregnenolone was converted primarily into 7 α - and/or 7 β -hydroxypregnenolone and allopregnanolone and/or epipregnanolone in the pineal gland [53]. Comparison of the production of these major neurosteroids by HPLC analysis and the expressions of their steroidogenic enzyme mRNAs by real-time PCR in the pineal gland revealed that the synthesis

of 7 α - and/or 7 β -hydroxypregnenolone and the expression of *Cyp7b* mRNA occur in both sexes of adult and juvenile quails, while they are significantly higher in juveniles than in adults [53]. Allopregnanolone and/or epipregnanolone synthesis and *Srd5a* mRNA expression were also higher in juveniles than in adults in both sexes [53].

Subsequently, the syntheses of these major neurosteroids and the expressions of their steroidogenic enzyme mRNAs in the pineal gland were compared with those of different brain regions. The synthesis of 7 α - and/or 7 β -hydroxypregnenolone and the expression of *Cyp7b* mRNA were higher in the pineal gland than in the cerebellum and diencephalon [53]. Allopregnanolone and/or epipregnanolone synthesis and *Srd5a* mRNA expression were also higher in the pineal gland than in the cerebellum and diencephalon [53]. It appears that the pineal gland produces 7 α - and/or 7 β -hydroxypregnenolone and allopregnanolone and/or epipregnanolone far more abundantly than other brain regions.

To investigate the release of neurosteroids from the pineal gland, the pineal glands of quail chicks were cultured in medium 199 and these major neurosteroids were analyzed by GC-MS. Significant amounts of 7 α -hydroxypregnenolone and allopregnanolone were found to be released from the pineal gland into the culture medium, unlike 7 β -hydroxypregnenolone and epipregnanolone [53] (fig. 1). Accordingly, 7 α -hydroxypregnenolone and allopregnanolone appear to be the major neurosteroids secreted from the pineal gland (fig. 1).

Biological Actions of Pineal Neurosteroids in the Brain

Biological Action of Pineal 7 α -hydroxypregnenolone on Locomotor Activity

The avian pineal gland is intrinsically light-sensitive, owing to its expression of several opsins including pinopsin [84], red-sensitive cone pigment [85], melanopsin [86] and neuropsin [87]. A combination of these opsins confers light-sensitivity on the pineal function governing rhythmic and nighttime-specific production of melatonin [88]. Hence, the avian pineal gland has been widely used for studies on light-regulation of the circadian clock [88]. The comprehensive analysis of light-regulated genes in the chicken pineal gland led to an unexpected finding that the pineal gland actively produces 7 α -hydroxypregnenolone [52].

The circadian clock is the internal time-measuring system that controls daily rhythms of physiology and behavior

even in the absence of external time cues. As an important feature of the circadian clock, it is entrained to the external day/night cycles, and therefore the phase of the circadian clock is adjusted by the changes of environmental conditions such as light, temperature, and food in a time-of-day-dependent manner [89]. For example, a light pulse given at early night and late night induce phase delay and advance, respectively, while the light at subjective day causes no significant phase shifting effect. Despite the importance of phase-dependent light response as seen in the conserved feature across species, its molecular mechanism still remains to be solved. The chick pineal gland has been considered as one of the best tissues to work on this issue [88]. The circadian clock genes, such as *Per*, *Cry*, *Clock*, and *Bmal*, are highly conserved between the chick and mouse, and the transcription- and translation-based feedback loop of the clock genes forms a core part of the circadian clock regulating rhythmic expression of a variety of genes. For example, expression of *AANAT* gene encoding the rate limiting enzyme of melatonin synthesis is regulated by CLOCK-BMAL1 through an E box element in the promoter [90]. Chicken pineal transcriptome analyses identified many rhythmic genes involved in a variety of physiological processes including melatonin synthesis [91, 92]. Among the clock (-related) genes, *E4bp4* encodes a basic leucine zipper transcription factor that represses *Per2* expression. *E4bp4* expression in the chick pineal gland is induced by a light pulse given at early night that causes the phase delay [93, 94] (fig. 2). To approach the molecular mechanism of the light-dependent phase-shift of the circadian clock, GeneChip analysis was conducted in order to search for genes induced by a light pulse at different time points of the day in the chicken pineal gland. Dark-reared juvenile chicks were compared with those exposed to light given at three time points of the day; daytime, early night or late night. The comprehensive transcriptome analysis revealed that *Insig-1* and *HMG-CoA synthase* genes show a light response pattern similar to that of *E4bp4* that is strongly induced by a light pulse given at early night [52]. *Insig-1* and *HMG-CoA synthase* genes are involved in cholesterol biosynthetic pathway and the targets of a transcription factor SREBP (sterol regulatory element-binding protein). Noticeably, the light pulse given at early night stimulated the formation of the active form of SREBP transcription factor and, consistently, induced a number of SREBP-target genes involved in cholesterol biosynthesis. In transcription reporter assays, SREBP activated the transcription from the chicken *E4bp4* promoter, indicating a novel role of SREBP in the photic input pathway of

Fig. 2. Light-stimulated production of 7 α -hydroxypregnenolone in the pineal gland. A light pulse given at early night activates formation of the active form of SREBP, sterol regulatory element-binding protein, resulting in induction of a number of genes governing cholesterol biosynthesis as well as a clock gene *E4bp4* [52]. E4BP4 is a transcription factor that regulates the circadian clock through repression of clock gene *Per2* [93, 94]. SREBP-mediated induction of a series of cholesterol biosynthetic genes would enhance cholesterol synthesis and lead to production of 7 α -hydroxypregnenolone, a neurosteroid stimulating locomotion. For details, see text.

the circadian clock activated at early night (fig. 2). In contrast, the transcriptome analysis uncovered an activation of the heat shock and endoplasmic reticulum stress response pathways at late night [52], a potential connection of which with the core clock mechanism needs to be further characterized.

The photic induction of a series of SREBP-target genes involved in cholesterol biosynthesis raised the possibility that production of cholesterol (and its derivatives) may be up-regulated by light in the pineal gland. Based on the observations that *StAR*, *P450scc*, and *P450 $_{7\alpha}$* genes in the pineal gland are induced by light (see above) and that the locomotor activity is enhanced by 7 α -hydroxypregnenolone in quail [66], neurosteroidogenesis in the chick pineal gland was investigated. Surprisingly, the chick pineal gland was found to have high activities to produce and secrete 7 α -hydroxypregnenolone [52] (fig. 2). In accordance with the transcriptional changes of cholesterol biosynthesis genes, a light pulse given at early night, but not at late night and daytime, activates 7 α -hydroxypregnenolone production. Furthermore, the light exposure stimulates the locomotor activity of dark-reared juvenile chicks more strongly at early night than the other time points. Intriguingly, pinealectomy (Px) reduces the light-dependent stimulation of the locomotor activity at early night. Collectively, the light activates the pineal production of 7 α -hydroxypregnenolone in a time-of-day-dependent manner under the control of the circadian clock, and these properties may be essential for regulation of locomotor activity (fig. 2). The pineal gland appears to regulate sleep/wake cycles through dual hormonal signals: rhythmic production of melatonin and light-dependent production of 7 α -hydroxypregnenolone.

Biological Action of Pineal Allopregnanolone on Purkinje Cell Survival

Because the two major pineal neurosteroids, 7 α -hydroxypregnenolone and allopregnanolone, are abundantly released from the pineal gland of juvenile birds [53], not only pineal 7 α -hydroxypregnenolone but also pineal allopregnanolone may play important roles in the brain of birds during development. In birds, the pineal gland is located near the cerebellum. The Purkinje cell is a principal cerebellar neuron that integrates the process of memory and learning. It has been reported that in birds and mammals Px induces cell loss in the brain including Purkinje cells during development [95, 96]. This observation suggests that allopregnanolone and/or 7 α -hydroxypregnenolone secreted by the pineal gland may be involved in Purkinje cell survival during development.

To test this hypothesis, we conducted a series of experiments in the male juvenile quail. Px decreased the concentration of allopregnanolone in the cerebellum and induced apoptosis of Purkinje cells, whereas administration of allopregnanolone to Px quail chicks increased the concentration of allopregnanolone in the cerebellum and prevented apoptosis of Purkinje cells [53]. We further indicated that pineal allopregnanolone reaches the cerebellar Purkinje cells by diffusion shown by injection of ^3H -allopregnanolone close to the pineal lumen [53] (fig. 3). Thus, allopregnanolone secreted by the pineal gland is considered to be an important factor for Purkinje cell survival during development (fig. 3). In contrast to allopregnanolone, administration of 7 α -hydroxypregnenolone to Px quail chicks did not increase Purkinje cell survival [53]. Although 7 α -hydroxypregnenolone did not facilitate Purkinje cell survival, this neurosteroid is involved in the reg-

Fig. 3. Neuroprotective action of pineal allopregnanolone on Purkinje cell survival. Allopregnanolone is exceedingly produced in the pineal gland compared with other brain regions, and may affect the adjacent cerebellar Purkinje cells by diffusion, and saves Purkinje cells from apoptosis in the juvenile quail. Secreted pineal allopregnanolone inhibits the expression of active caspase-3 that facilitates apoptosis of Purkinje cells in the cerebellum during development. For details, see text.

ulation of locomotion in birds [52, 66] as mentioned above.

The induction of cell loss of Purkinje cells in the cerebellum by Px suggests that certain other component(s) in the pineal gland may contribute to Purkinje cell survival during development. However, pineal melatonin did not facilitate Purkinje cell survival during development and did not affect cerebellar allopregnanolone synthesis and circulating allopregnanolone level in quail chicks [53]. It is therefore considered that pineal allopregnanolone but not melatonin acts as an important component of the pineal gland for Purkinje cell survival during development. We found that a significant amount of allopregnanolone is released from cultured pineal glands of juvenile quail [53]. This result suggests that pineal allopregnanolone reaches the target sites within the cerebellum by diffusion. Nevertheless, we cannot exclude the possibility of an anatomical link (e.g. blood vessels) from the pineal gland to the cerebellum.

Finally, we investigated the factor that mediates the neuroprotective effect of pineal allopregnanolone in Purkinje cells. It is well known that caspase-3 plays an important role in Purkinje cell death in vertebrates [97, 98]. Caspase-3 is a crucial mediator of apoptosis [97] in vertebrates including birds [98, 99]. Interestingly, Px increased the number of Purkinje cells that expressed active cas-

pase-3, a key protease in apoptotic pathway, in quail chicks and administration of allopregnanolone to Px quail chicks decreased the number of Purkinje cells expressing active caspase-3 [53]. These findings indicate that the neuroprotective effect of pineal allopregnanolone on Purkinje cells is associated with the decrease in caspase-3 activity during development. Accordingly, pineal allopregnanolone exerts antiapoptotic effects in Purkinje cells by suppressing the activity of caspase-3 during development (fig. 3). This is a new function of the pineal gland for the prevention of Purkinje cell death in the developing cerebellum.

It is unclear whether the action of pineal allopregnanolone on caspase-3 activity in the Purkinje cell is rapid (*i.e.* mediated through a membrane receptor) or slow (*i.e.* involving transcriptional activation). On the other hand, the action of allopregnanolone produced in the brain is likely mediated through interaction with the GABA_A receptor pathway, since allopregnanolone is a potent allosteric modulator of GABA_A receptors [100, 101]. However, the intracellular signaling pathway of pineal allopregnanolone suppressing the activity of caspase-3 in the Purkinje cell remains unclear. Future studies are needed to clarify the intracellular signaling pathway of pineal allopregnanolone in the Purkinje cell.

Conclusions and Future Directions

It was generally accepted that the pineal gland, an endocrine organ located close to the brain, transduces photoperiodic changes to the neuroendocrine system by executing rhythmic secretion of melatonin. However, the formation of neurosteroids in the pineal gland was, until recently, unknown in vertebrates. Our recent studies provide new evidence that the pineal gland actively produces neurosteroids *de novo* from cholesterol. This is a paradigm shift of neurosteroid formation, because it was generally believed that neurosteroids are produced only in neurons and glial cells in the central and peripheral nervous systems. Importantly, 7 α -hydroxypregnenolone is a major pineal neurosteroid that stimulates locomotor activity of juvenile birds, connecting light-induced gene expression with locomotion. The other major pineal neurosteroid allopregnanolone prevents cell death of Purkinje cells by suppressing the activity of caspase-3 during cerebellar development. In birds, the syntheses of 7 α -hydroxypregnenolone and allopregnanolone in the pineal gland are higher in juveniles than in adults, suggesting that these pineal neurosteroids may not be involved in the control of locomotor activity and Purkinje

cell survival in adults. However, further studies are needed to determine physiological functions of these two major neurosteroids in adults. Unlike birds, it remains unclear whether the pineal gland of mammals produces neurosteroids including 7 α -hydroxypregnenolone and allopregnanolone. Further studies are needed to demonstrate the biosynthesis and biological actions of pineal neurosteroids in mammals. These are new functions of the pineal gland by the actions of pineal neurosteroids.

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The authors have nothing to disclose.

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