INTRODUCTION

The brain has traditionally been considered as a target site for peripheral steroid hormones. In addition to this classical concept, new findings over the past two decades have shown that the brain has the capacity to synthesize steroids de novo from cholesterol, the so-called “neurosteroids” (for reviews, see Baulieu, 1997; Tsutsui et al., 1999, 2000, 2003, 2006; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Do-Rego et al., 2009a). Brain neurosteroid contents are not affected by removal of peripheral steroid hormones following adrenalectomy, castration, and/or hypophysectomy (Corpéchot et al., 1981, 1983; Tsutsui and Yamazaki, 1995; Mensah-Nyagan et al., 1996a) and diurnal and seasonal changes in neurosteroid contents are evident in the brain (Takase et al., 1999; Matsunaga et al., 2004; Tsutsui et al., 2008; Haraguchi et al., 2010).

The formation of neurosteroids in the brain was originally demonstrated in mammals by Baulieu and colleagues (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Lanthier and Patwardhan, 1986; Robel et al., 1987; Jo et al., 1989; Mathur et al., 1993; Mellon and Deschepper, 1993; Compagnone et al., 1995). In non-mammalian vertebrates, i.e., in birds, amphibians, and fish, the brain expresses several kinds of steroidogenic enzymes and produces a variety of neurosteroids (for reviews, see Tsutsui et al., 1999, 2000, 2003, 2006; Mellon and Vaudry, 2001; Tsutsui and Mellon, 2006; Do-Rego et al., 2009a). Birds have always served as excellent animal models for understanding the actions of steroids on brain and behavior, and studies investigating neurosteroid synthesis and action in birds may be of general significance in vertebrates. We therefore analyzed neurosteroids formed from cholesterol in the avian brain using the Japanese quail Coturnix japonica (Tsutsui and Yamazaki, 1993; Usui et al., 1995; Tsutsui et al., 1997, 1999, 2003, 2004; Ukena et al., 1999, 2001; Matsunaga et al., 2004; Tsutsui and Schlinger, 2001). Schlinger and colleagues undertook similar studies in passeriform bird species (Vanson et al., 1996; Schlinger et al., 1999; Freking et al., 2000; Soma et al., 2004). The formation of several neurosteroids from cholesterol is now also well documented in amphibians (Mensah-Nyagan et al., 1994, 1996a,b; 1999; Beauchan et al., 1999; Takase et al., 1999, 2002; Inai et al., 2003; Matsunaga et al., 2004; Do-Rego et al., 2007; Bruzzone et al., 2010) and fish (Sakamoto et al., 2001). Accordingly, de novo neurosteroidogenesis in the brain from cholesterol appears to be a conserved property across vertebrates (for reviews, see Baulieu, 1997; Tsutsui et al., 1999, 2000, 2003, 2006; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Tsutsui and Mellon,
2006; Do-Rego et al., 2009a). However, the biosynthetic pathways leading to the formation of neurosteroids in vertebrates was not fully characterized (for a review, see Tsutsui et al., 2006).

In fact, we recently found that the new brain actively produces 7α-hydroxy pregnenolone, a previously undescribed amphibian neurosteroid, from pregnenolone (Matsunaga et al., 2004). Interestingly, 7α-hydroxy pregnenolone acts as a novel neuronal modulator to stimulate locomotor activity of newts (Matsunaga et al., 2004). We also identified 7α- and 7β-hydroxy pregnenolone in quail brain by using biochemical techniques (Tsutsui et al., 2008). It was subsequently shown that 7α-hydroxy pregnenolone, but not 7β-hydroxy pregnenolone, stimulates locomotor activity in quail (Tsutsui et al., 2008). Finally, we found that cytochrome P450α7α catalyzes the conversion of pregnenolone to 7α-hydroxy pregnenolone in the brain of these vertebrates (Tsutsui et al., 2008; Haraguchi et al., 2010).

This review describes the discovery of 7α-hydroxy pregnenolone, a new key regulator of locomotor activity in vertebrates, the mode of action, and the functional significance of this neurosteroid. This review also summarizes the current knowledge regarding the diurnal and seasonal changes in 7α-hydroxy pregnenolone synthesis, and their regulatory mechanisms.

**OVERVIEW OF NEUROSTEROIDOGENESIS IN THE BRAIN**

It has long been established that the central nervous system is a target site for steroid hormone action. More recently, several laboratories have established unequivocally that the brain has the ability to synthesize neurosteroids from cholesterol. The new concept that neurosteroids could be formed de novo in the brain of mammals was first put forward by Baulieu and colleagues (for a review, see Baulieu, 1997). These observations revealed that the brain of mammals can synthesize neurosteroids de novo from cholesterol (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Robel et al., 1986, 1987; Jo et al., 1989; Mathur et al., 1993). They also showed that the brain content of these steroids remains constant even after removal of peripheral steroids by adrenalectomy, castration, and/or hypophysectomy. These observations revealed that the brain of mammals can synthesize neurosteroids de novo from cholesterol (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Robel et al., 1986, 1987; Jo et al., 1989). Following this pioneering discovery in the brain of mammals (for a review, see Baulieu, 1997), the concept of de novo neurosteroidogenesis from cholesterol was extended to the brain of birds (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Vanson et al., 1996; Tsutsui et al., 1997, 1999, 2003; Schlinger et al., 1999; Ukena et al., 1999, 2001; Freking et al., 2000; Lea et al., 2001; Matsunaga et al., 2001, 2002; Tsutsui and Schlinger, 2001; Soma et al., 2004), and amphibia (Mensah-Nyagan et al., 1994, 1996a, b, 1999; Beaufjean et al., 1999; Takase et al., 1999, 2002; Inai et al., 2003; Matsunaga et al., 2004; Do-Rego et al., 2007; Bruzzone et al., 2010).

In peripheral organs, including the gonads, adrenals, and placenta, pregnenolone is the common precursor of all steroid hormones. The formation of pregnenolone is initiated by cleavage of the cholesterol side-chain by cytochrome P450scc, a rate-limiting mitochondrial enzyme (Figure 1). As an initial step in the demonstration of pregnenolone biosynthesis in the brain of birds, Tsutsui and Yamazaki (1995) showed that the concentration of pregnenolone in the quail brain is higher than in the plasma. The accumulation of pregnenolone in the quail brain was largely independent of peripheral steroidogenic organs since a high level of brain pregnenolone persists in hypophysectomized birds (Tsutsui and Yamazaki, 1995). The formation of pregnenolone from cholesterol was found to occur in quail brain mitochondria, and Western immunoblot analysis with an antibody against purified bovine P450scc confirmed the presence of the P450scc protein in quail brain pregnenolone. Although the quail brain possesses both 7α- and 7β-hydroxy pregnenolone, it is still unclear whether P450α7α can also convert pregnenolone to 7β-hydroxy pregnenolone. See the text for details.

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**FIGURE 1** | Biosynthetic pathways for neurosteroids in the quail brain.

The black arrows indicate the biosynthetic pathways of neurosteroids identified previously in the quail brain. The red arrow indicates a newly identified biosynthetic pathway resulting in the biosynthesis of 7α-hydroxy pregnenolone.
brain homogenates (Tsutsui and Yamazaki, 1995). Similar findings were reported in the ring dove (Tsutsui et al., 1999; Lea et al., 2001) and zebra finch (Frequing et al., 2000). Taken together, these data indicate that, in birds, the brain contains cytochrome P450scc and synthesizes pregnenolone from cholesterol (for reviews, see Tsutsui et al., 1997, 1999, 2003, 2006; Tsutsui and Schlinger, 2001) as previously shown is mammals (Corpechot et al., 1981, 1983; Robel and Baulieu, 1985; Lanthier and Patwardhan, 1986; Robel et al., 1986, 1987; Hu et al., 1987; Le Goascogne et al., 1987; Jo et al., 1989; Jung-Testas et al., 1989; Baulieu and Robel, 1990; Iwahashi et al., 1990; Baulieu, 1991; Papadopoulos et al., 1992; Mathur et al., 1993; Mellon and Deschepper, 1993; Compagnone et al., 1995; Kohchi et al., 1998; Ukena et al., 1998).

In the brain of birds, pregnenolone is metabolized to progesterone by 3β-hydroxysteroid dehydrogenase/Δ5-Δ4-isomerase (3β-HSD; Figure 1). By using biochemical techniques combined with high-performance liquid chromatography (HPLC) analysis, we have demonstrated that, in the quail brain, pregnenolone is converted to progesterone (Ukena et al., 1999). The biosynthesis of progesterone increased with time and is completely abolished by triostane, a specific inhibitor of 3β-HSD (Ukena et al., 1999). The expression of 3β-HSD mRNA in the quail brain was revealed by using RT-PCR analysis together with Southern hybridization (Ukena et al., 1999). Similar observations were reported in the brains of zebra finches (Vanson et al., 1996) and ring doves (Lea et al., 2001). The expression of both 3β-HSD protein and its mRNA has also been observed in the brain of mammals (Dupont et al., 1994; Guennoun et al., 1995; Sanne and Krueger, 1995; Kohchi et al., 1998; Ukena et al., 1999). In addition, 3β-HSD activity has been demonstrated biochemically in the brain of mammals (Weidenfeld et al., 1980; Akwa et al., 1993; Kabbadj et al., 1993; Ukena et al., 1999).

Progesterone is further metabolized via 5β reduction to several derivatives including 5β-dihydropregesterone (5β-DHP) and 3β,5β-tetrahydroprogesterone (3β,5β-THP; Figure 1). Both 5β-DHP (Ukena et al., 2001) and 3β,5β-THP (Tsutsui et al., 2003) have been shown to occur in the quail brain. In birds, 5β-reduction also represents a route of androgen metabolism in the brain (Massa and Sharp, 1981; Schlinger and Callard, 1987). In contrast to birds, in mammals, progesterone is converted to 5α-DHP and 3α,5α-THP due to the presence of 5α-reductase and 3α-HSD (for reviews, see Baulieu, 1997; Compagnone and Mellon, 2000). Another route of progesterone metabolism is mediated by 17α-hydroxylase/c17,20-lyase (cytochrome P450 17α,20β-HSD) which, in addition to converting pregnenolone to dehydroepiandrosterone via 17α-hydroxypregnenolone, also converts progesterone to androstenedione via 17α-hydroxyprogesterone (Figure 1). Both of these metabolic pathways have been demonstrated in the quail brain using biochemical techniques combined with HPLC analysis, and by RT-PCR analysis of cytochrome P450 17α,20β-HSD mRNA (Matsunaga et al., 2001, 2002). The expression of P450 17α,20β-HSD has also been detected in the brain of mammals (Compagnone et al., 1995; Strömstedt and Waterman, 1995; Kohchi et al., 1998).

The avian brain has also been shown to contain 17β-hydroxysteroid dehydrogenase (17β-HSD) that is needed to convert androstenedione to testosterone, and cytochrome P450arom, which converts testosterone to estradiol-17β (Figure 1). The expression and localization of 17β-HSD in the quail brain was demonstrated by Matsunaga et al. (2002) while other studies revealed the expression and localization of cytochrome P450arom (Schlinger and Callard, 1987, 1989a,b, 1991; Balthazart et al., 1990a,b, 1991). Therefore, not only androgens but also estrogens may be synthesized directly in the avian brain.

There is also now clear evidence that the brain of amphibians has the capability of synthesizing neurosteroids and the presence of steroidogenic enzymes has been documented in the brain of both anurans (frogs) and urodeles (newts; for reviews, see Mensah-Nyagan et al., 1999, 2000, 2009b, 2010; Mellon and Vaudry, 2001; Do-Rego et al., 2009a,b). In Xenopus laevis, Takase et al. (1999) have reported that the concentrations of pregnenolone, the main precursor of neurosteroids, and its sulfate ester are higher in the brain than in the gonad and plasma (Takase et al., 1999). In the European green frog Rana ridibunda, incubation of brain slices with tritiated pregnenolone combined with HPLC analysis of the incubation medium has shown that androgens, estrogens, and adrenal steroids are generated from pregnenolone de novo (Mensah-Nyagan et al., 1994, 1996a,b). Concurrently, immunohistochemical studies revealed the presence of various steroidogenic enzymes in the brain of Rana species, such as cytochrome P450scc (Takase et al., 1999), 3β-HSD (Mensah-Nyagan et al., 1994), cytochrome P450 17α,20β-HSD (Do-Rego et al., 2007), hydroxysteroid sulfotransferase (Beaujean et al., 1999), 17β-HSD (Mensah-Nyagan et al., 1996b), and 5α-reductase (Bruzzone et al., 2010). Takase et al. (1999) have also reported that the brain of R. nigromaculata also expresses mRNA encoding cytochrome P450 17α-lyase, which catalyzes the final step of biosynthesis of the adrenal steroids, corticosterone, and aldosterone (Takase et al., 2002). In urodeles, Inai et al. (2003) and Takase et al. (2010) have found that the brain of the newt Cynops pyrrhogaster expresses cytochrome P450scc and produces pregnenolone from cholesterol. In this species, the tissue concentration of pregnenolone was higher in the brain than in peripheral steroidogenic organs. Inai et al. (2003) have found that the newt brain also expresses 3β-HSD and produces progesterone from pregnenolone. It thus appears that biosynthesis of neurosteroids occurs in the brain of both anuran and urodele species.

**Identification of 7α-HydroxyPregnenolone in the Brain**

We initially found that the brain of the newt C. pyrrhogaster actively produces an unknown neurosteroid from pregnenolone. It appeared that the concentration of this compound is greater than those of any neurosteroids identified previously in amphibians, suggesting that this unknown neurosteroid could be involved in key neurophysiological functions. Therefore, we sought to identify this amphibian neurosteroid from the newt brain by using biochemical and analytical techniques (Matsunaga et al., 2004). Incubation of newt brain homogenates with tritiated pregnenolone combined with HPLC analysis of the incubation medium has shown that a major radioactive peak is detected before pregnenolone elution (Matsunaga et al., 2004). Using several non-radioactive steroids as reference standards for HPLC analysis, Matsunaga et al. (2004) have indicated that 7α- and 7β-hydroxypregnenolone exhibit the same retention time as the radioactive peak. The radioactive HPLC peak corresponding to
7α- and 7β-hydroxyprogrenolone increased in a time-dependent manner, and the inhibitor of cytochrome P450s, ketoconazole, reduced the production of the metabolite (Matsunaga et al., 2004). This HPLC peak was collected and subjected to thin-layer chromatography (TLC; Matsunaga et al., 2004). Only 7α-hydroxyprogrenolone had the same retention position as the radioactive metabolite of progrenolone under identical chromatographic conditions (Matsunaga et al., 2004). The progrenolone metabolite was further analyzed by gas chromatography–mass spectrometry (GC–MS). Trimethylsilyl ether derivatives of the authentic 7α- and 7β-hydroxyprogrenolone and the metabolite obtained from non-radioactive progrenolone were prepared and subsequently applied to GC–MS analysis (Matsunaga et al., 2004). Although 7α- and 7β-hydroxyprogrenolone had the same mass spectrum, their retention times were different in GC–MS. Based on GC–MS, 7α-hydroxyprogrenolone and the metabolite had an identical retention time and the same diagnostically important ions. Thus, we could identify the unknown neurosteroid converted from progrenolone in the newt brain as 7α-hydroxyprogrenolone (Matsunaga et al., 2004).

Subsequently, we found 7α- and 7β-hydroxyprogrenolone in the quail brain by using the same biochemical techniques (Tsutsui et al., 2008). Quail brain homogenates were incubated with tritiated progrenolone, and radioactive metabolites were analyzed by reversed-phase HPLC (Tsutsui et al., 2008). A major radioactive peak of the metabolites was collected and subjected to TLC (Tsutsui et al., 2008). Quail brain homogenates produced two radioactive metabolites from 3H-progrenolone that exhibit the same retention times as the 7α- and 7β-hydroxyprogrenolone standards. The metabolites of progrenolone were further analyzed by GC–MS (Tsutsui et al., 2008). The metabolites had retention times that are identical to 7α- and 7β-hydroxyprogrenolone. The unknown avian neurosteroids formed from progrenolone in the quail brain were thus identified as 7α- and 7β-hydroxyprogrenolone (Figure 1; Tsutsui et al., 2008).

**FIGURE 1** 7α-Hydroxyprogrenolone synthesis in COS-7 cells expressing quail P450α. (A) HPLC profile of 7α- and 7β-hydroxyprogrenolone and progrenolone extracted from COS-7 cells that were transfected with quail P450α cDNA and incubated with 3H-progrenolone. The ordinate indicates the radioactivity measured in each HPLC fraction, and the arrows indicate the elution positions of standard steroids, i.e., progrenolone and 7α- and/or 7β-hydroxyprogrenolone. (B) HPLC profile of the extract from COS-7 cells that were transfected with quail P450α cDNA and incubated with 3H-progrenolone in the presence of 10−4 M ketoconazole, an inhibitor of P450s. (C) HPLC profile of the extract from non-transfected COS-7 cells incubated with 3H-progrenolone. (D) GC–MS analysis of 7α-hydroxyprogrenolone. GC–MS total-ion current (TIC) trace of the extract from COS-7 cells that were transfected with quail P450α cDNA and incubated with 10−2 M progrenolone. The arrowheads show the peaks corresponding to 7α-hydroxyprogrenolone and progrenolone. (E) GC–MS TIC trace of the extract from non-transfected COS-7 cells. (F) GC–MS of trimethylsilyl ether derivatives of an unknown progrenolone metabolite and authentic 7α-hydroxyprogrenolone. The arrowheads indicate diagnostically important ions of 7α-hydroxyprogrenolone (m/z 386 and 476). Adapted from Tsutsui et al. (2008).

**IDENTIFICATION OF CYTOCHROME P450α PRODUCING 7α-HYDROXYPROGRENOLONE IN THE BRAIN**

7α-Hydroxyprogrenolone is synthesized from progrenolone through the enzymatic activity of cytochrome P450α (Figures 1 and 2). In order to prove that 7α-hydroxyprogrenolone is synthesized in the brain, it is therefore necessary to show that the brain expresses P450α. A full length, 2341 bp cDNA prepared from quail brain tissue was identified as encoding a putative cytochrome P450α (Tsutsui et al., 2008). The putative quail P450α open reading frame was initiated with a methionine at...
nucleotide 72 and terminates with a TGA codon at nucleotide 1581, encoding a protein of 503 amino acids. The enzymatic activity of this putative quail P450α was demonstrated in homogenates of COS-7 cells transfected with the putative quail P450α cDNA (Figure 2; Tsutsui et al., 2008). As demonstrated by HPLC analyses, the homogenate converted pregnenolone to 7α- and/or 7β-hydroxyprogrenenolone (Figure 2A), and ketoconazole partially inhibited this conversion (Figure 2B). Pregnenolone was not converted to 7α- and/or 7β-hydroxyprogrenenolone in COS-7 cells without transfection of quail P450α cDNA (Figure 2C). Subsequently, 7α-hydroxyprogrenenolone but not 7β-hydroxyprogrenenolone synthesis was confirmed by GC–MS (Figures 2D–F; Tsutsui et al., 2008). Although it is still unclear whether P450α can also convert pregnenolone to 7β-hydroxyprogrenenolone, the presence of 7β-hydroxyprogrenenolone as well as 7α-hydroxyprogrenenolone is evident in the quail brain (Figure 1; Tsutsui et al., 2008). The production of 7α-hydroxyprogrenenolone in the brain may be a conserved property of vertebrates, because this neurosteroid has also been identified in the brains of newts (Matsunaga et al., 2004) and mammals (Akwa et al., 1992; Doostzadeh and Morfin, 1997; Weill-Engerer et al., 2003; Yau et al., 2003).

Subsequently, a cDNA encoding a putative cytochrome P450α was identified from newt brain tissue (Haraguchi et al., 2010). The newt P450α cDNA had a full length of 2598 bp. The open reading frame commenced with a methionine at nucleotide 157 and terminates with a TAG codon at nucleotide 1681, encoding a protein of 503 amino acids. The enzymatic activity of this putative newt P450α was then demonstrated (Haraguchi et al., 2010). The homogenate of COS-7 cells transfected with the putative newt P450α cDNA converted pregnenolone into 7α-hydroxyprogrenenolone as shown by HPLC analysis, and ketoconazole abolished this metabolic process. COS-7 cells without transfection of newt P450α cDNA did not convert pregnenolone into 7α-hydroxyprogrenenolone. 7α-Hydroxyprogrenenolone synthesis was further confirmed by GC–MS analysis (Haraguchi et al., 2010).

**BIOLOGICAL ACTION OF 7α-HYDROXYPREGNENOLONE ON LOCOMOTOR ACTIVITY**

Because 7α-hydroxyprogrenenolone is actively produced in the brain of the newt C. pyrrhogaster, this seasonally breeding amphibian has served as a suitable animal model to investigate the biological action of 7α-hydroxyprogrenenolone. The production of 7α-hydroxyprogrenenolone in the diencephalon and rhombencephalon of male newts was much higher than in the telencephalon and peripheral steroidogenic glands (Matsunaga et al., 2004). In addition, 7α-hydroxyprogrenenolone synthesis in the brain of male newts showed marked changes during the annual breeding cycle, with a maximum level in the spring breeding period when locomotor activity of wild populations of the same species increases (Matsunaga et al., 2004; see Seasonal Changes in 7α-Hydroxyprogrenenolone Synthesis and Locomotor Activity and their Regulatory Mechanisms for further discussion). We therefore analyzed the effect of 7α-hydroxyprogrenenolone on locomotor activity (Matsunaga et al., 2004).

Behavioral analysis demonstrated that administration of 7α-hydroxyprogrenenolone acutely increases locomotor activity of male newts in the non-breeding period when endogenous 7α-hydroxyprogrenenolone synthesis in the brain is low (Figure 3; Matsunaga et al., 2004). This stimulatory effect occurred in a dose-dependent manner with a threshold dose ranging from 0.5 to 1 ng through intracerebroventricular (i.c.v.) injection (Figure 3), corresponding to the physiological range observed in the brain of normal newts (Matsunaga et al., 2004). Therefore, 7α-hydroxyprogrenenolone may act as a novel neuronal modulator to stimulate locomotor activity of male newts, and the increase in locomotor activity of male newts that occurs during the spring breeding period may be ascribed to an increase in the production of 7α-hydroxyprogrenenolone.

Because the male quail displays a robust locomotor activity rhythm when held under typical light/dark lighting schemes (Wilson, 1972; Wada, 1979; see Diurnal Changes in 7α-Hydroxyprogrenenolone Synthesis and Locomotor Activity and their Regulatory Mechanisms for further discussion), this bird has also served as an appropriate animal model to investigate the biological action of 7α- and 7β-hydroxyprogrenenolone. Both neurosteroids were therefore administered i.c.v. to male quail during night, when activity is low, to examine whether they affect locomotor activity (Tsutsui et al., 2008; see Diurnal Changes in 7α-Hydroxyprogrenenolone Synthesis and Locomotor Activity and their Regulatory Mechanisms for further discussion). A stimulatory dose-dependent effect of 7α-hydroxyprogrenenolone was observed with effective doses ranging between 10 and 100 ng (Tsutsui et al., 2008). In contrast, even at the highest dose tested (100 ng), 7β-hydroxyprogrenenolone did not

![FIGURE 3](Image 335x159 to 519x393)

**FIGURE 3** Effect of 7α-hydroxyprogrenenolone on locomotor activity of male newt. Male newts in the non-breeding period received an i.c.v. injection of vehicle (n = 7), 7α-hydroxyprogrenenolone (7α-OH PREG; 0.1 and 0.5 ng: n = 6; 1 ng: n = 7), or 7β-hydroxyprogrenenolone (7β-OH PREG; 1 ng: n = 6). Each column and vertical line represents the mean ± SEM total number of crossings. **p < 0.01 vs. vehicle, †p < 0.05 vs. 0.1 ng of 7α-hydroxyprogrenenolone injection by one-way ANOVA, followed by Duncan’s multiple range test. Adapted from Matsunaga et al. (2004).
influence locomotor activity (Tsutsui et al., 2008). It thus appears that 7α-hydroxypregnenolone, but not 7β-hydroxypregnenolone, acts as a neuronal modulator to stimulate locomotor activity in male quail.

**MODE OF ACTION OF 7α-HYDROXYPREGNENOLONE ON LOCOMOTOR ACTIVITY**

To investigate the mode of action of 7α-hydroxypregnenolone on locomotion, the concentrations of several monoamines (norepinephrine, epinephrine, dopamine, and 5-hydroxytryptamine) were measured by HPLC-electrochemical detection (ECD) 5 min after an i.c.v. injection of 7α-hydroxypregnenolone to non-breeding male newts (Matsunaga et al., 2004). 7α-Hydroxypregnenolone significantly increased the concentration of dopamine in the male newt brain, particularly in the rostral brain region including the striatum, which is known to be involved in the regulation of locomotor behavior (Matsunaga et al., 2004). In contrast, there were no significant differences in the concentrations of other monoamines, i.e., norepinephrine, epinephrine, and 5-hydroxytryptamine (Matsunaga et al., 2004). In vitro experiments further revealed that 7α-hydroxypregnenolone treatment results in a concentration-dependent increase in the release of dopamine from cultured male newt brain tissue after a 10-min incubation (Matsunaga et al., 2004). The threshold concentration ranged between 10^{-8} and 10^{-7} M (Matsunaga et al., 2004). The effect of 7α-hydroxypregnenolone on locomotion was abolished by administration of haloperidol or sulpiride, two dopamine D_{1} receptor antagonists (Matsunaga et al., 2004). In contrast, the dopamine D_{1} receptor antagonist SCH23390 did not block the effect of 7α-hydroxypregnenolone (Matsunaga et al., 2004). These results indicate that the stimulatory effect of 7α-hydroxypregnenolone on locomotor activity is mediated through dopamine D_{1} receptors. To recapitulate, 7α-hydroxypregnenolone synthesized actively in the diencephalon and rhombencephalon, by acting on dopaminergic neurons localized in the posterior tuberal (PT) nucleus and ventral tegmental area (VTA), may induce dopamine release from their terminals in the rostral brain region, notably in the striatum and nucleus accumbens (NA), and consequently increase locomotor activity of newts (Figure 4; Matsunaga et al., 2004).

To identify the cells producing 7α-hydroxypregnenolone in the quail brain, we investigated the expression of P450α2 by in situ hybridization. In the male diencephalon, the expression of P450α2 mRNA was localized in the nucleus preopticus medialis (POM), the nucleus paraventricularis magnocellularis (PVN), the nucleus ventromedialis hypothalami (VMN), the nucleus dorsolateralis anterior thalami (DLA), and the nucleus lateralis anterior thalami (LA; Tsutsui et al., 2008). In quail as in newt (Matsunaga et al., 2004), 7α-hydroxypregnenolone increased the concentration of dopamine in the telencephalic region that encompasses the striatum (Sanberg, 1983; Sharp et al., 1987; Bardo et al., 1990). In birds, dopaminergic neurons that are located in the mesencephalic region, including the ventral tegmental area (VTA) and the substantia nigra (SN), project to the telencephalon notably the striatum (Mezey and Csillag, 2002; Hara et al., 2007). Interestingly, in birds as in mammals, the telencephalic region is enriched with dopamine D_{1} and D_{2} receptors (Ball et al., 1995; Levens et al., 2000). Thus, the stimulatory effect of 7α-hydroxypregnenolone on locomotor activity in male quail may be mediated by the dopaminergic system as previously shown in male newt. In sum, 7α-hydroxypregnenolone synthesized actively in the diencephalon, by acting on dopamine neurons localized in the VTA and SN, may induce dopamine release from their termini in the striatum, and consequently increase locomotor activity in male quail.

The fact that 7α-hydroxypregnenolone acutely increases locomotor activity in newt and quail suggests that the neurosteroid may act through a non-genomic rather than a genomic mechanism. In rat, the progesterone metabolite 3α,5α-THP (allopregnanolone) exerts its effects on locomotion (Wieland et al., 1995) and dopamine release (Bullock et al., 1997; Rougé-Pont et al., 2002) via a non-genomic pathway. Allopregnanolone may act through modulation of GABA_{A} receptors, since allopregnanolone is a potent allosteric modulator of GABA_{A} receptors (Paul and Purdy, 1992; Lambert et al., 1995) and dopaminergic neurons are regulated by GABAergic transmission (Laviolette and van der Kooy, 2001). Whether the acute actions of 7α-hydroxypregnenolone on dopamine release and locomotor activity in newt and quail are mediated through GABA_{A} receptors remain to be determined.

**SEX DIFFERENCES IN 7α-HYDROXYPREGNENOLONE SYNTHESIS AND LOCOMOTOR ACTIVITY**

In birds (Tsutsui et al., 2008) as in other vertebrates (Tsutsui, 1931; Iwata et al., 2000), the locomotor activity of males is known to be higher than that of females (Figure 5A). In quail, the production and concentration of 7α-hydroxypregnenolone in the male diencephalon were much higher than in female (Figures 5B,C; Tsutsui et al., 2008). Such a sexual dimorphism only occurs in the diencephalon (Tsutsui et al., 2008). There are similar sex differences in 3β-HSD and P450arom in the avian brain (Schlinger and Callard, 1987; Soma et al., 2004; Tam and Schlinger, 2007). In view of the sex difference in 7α-hydroxypregnenolone biosynthesis and concentration in the quail diencephalon (Figures 5B,C; Tsutsui et al., 2008), it seemed possible that this neurosteroid actively plays a role in the control of locomotor activity only in males. (Tsutsui et al., 2008) In support of this notion, administration of the P450 inhibitor ketoconazole in male quail decreased locomotor activity (Tsutsui et al., 2008). Unlike
males, 7α-hydroxy pregnenolone administration did not increase locomotor activity in females (Tsutsui et al., 2008). This observation suggests that the receptor for 7α-hydroxy pregnenolone is not present or is otherwise inactive in the female. In addition, the rate of 7α-hydroxy pregnenolone biosynthesis and the tissue concentration of 7α-hydroxy pregnenolone were significantly lower in the female quail diencephalon than in male (Figures 5B, C; Tsutsui et al., 2008). These data suggest that 7α-hydroxy pregnenolone may not affect locomotor activity in the female.

In newt, the biosynthesis and concentration of 7α-hydroxy pregnenolone in the male brain were also higher than in the female (Figures 5A, B; Matsunaga et al., 2004; Haraguchi et al., 2010). It is well known that sexually mature male newts in the breeding period move around much more than the females, searching sexually mature female partners or courting females prior to sperm transfer (Tsutsui, 1931; Iwata et al., 2000). It is therefore possible that, in newt as in quail, 7α-hydroxy pregnenolone may specifically affect the activity of the male brain. Taken together, these observations suggest that 7α-hydroxy pregnenolone may play a crucial role in the control of sex-dependent locomotor activity in vertebrates.

**DIURNAL AND SEASONAL CHANGES IN 7α-HYDROXYPREGNENOLOLE SYNTHESIS AND LOCOMOTOR ACTIVITY AND THEIR REGULATORY MECHANISMS**

To further investigate the functional significance of 7α-hydroxy pregnenolone in the regulation of locomotor activity, the correlation between locomotor activity and the concentrations of diencephalic 7α-hydroxy pregnenolone was studied in male quail exposed to daily photoperiods of 16:8 h light/dark (LD; lights on at 07:00 am, off at 11:00 pm). Locomotor activity of males was much higher than that of females from the time of lights on until noon, but thereafter decreased to female levels (Figure 5A; Tsutsui et al., 2008). In males, these changes in locomotor activity were correlated with concentrations of diencephalic 7α-hydroxy pregnenolone, the maximum value occurring at 11:00 am when locomotor activity was high (Figures 5A, B; Tsutsui et al., 2008). The functional significance of this correlation is supported by the observation that administration of ketoconazole, an inhibitor of P450s, inhibits locomotor activity at 11:00 am (Tsutsui et al., 2008). Thus, the increase in diencephalic 7α-hydroxy pregnenolone may be responsible, at least in part, for the higher locomotor activity in males. As mentioned in Section “Sex Differences in 7α-Hydroxy pregnenolone Synthesis and Locomotor Activity,” the low level of 7α-hydroxy pregnenolone biosynthesis and concentration in the female diencephalon suggests that this neurosteroid may not play a role in female locomotor activity (Figures 5A–C).

Further studies were undertaken to elucidate the mechanism regulating diurnal changes in 7α-hydroxy pregnenolone biosynthesis and 7α-hydroxy pregnenolone-dependent locomotor activity. Melatonin is known to be also involved in the regulation of locomotor activity in birds (Binkley et al., 1971; John et al., 1978; Cassone and Menaker, 1984; Chabot and Menaker, 1992; Hau and Gwinner, 1994; Warren and Cassone, 1995; Murakami et al., 2001), which suggested that melatonin may regulate diencephalic 7α-hydroxy pregnenolone biosynthesis, and thereby influence locomotor activity. To test this hypothesis, experiments involving melatonin manipulation were performed in male quail. Combination of pinealectomy (Px) and orbital enucleation (Ex) increased after 1 week the production and concentration of 7α-hydroxy pregnenolone (Figures 6A, B) and the expression of P450s in the quail diencephalon (Figure 6C). Conversely, melatonin administration to Px/Ex quail decreased the production and concentration of 7α-hydroxy pregnenolone and the expression of P450s in the diencephalon (Figures 6A–C; Tsutsui et al., 2008). Further, the inhibitory effect of melatonin on 7α-hydroxy pregnenolone synthesis was abolished by luzindole, a melatonin receptor antagonist (Tsutsui et al., 2008). These data indicate that melatonin acts to reduce P450a, expression through melatonin receptor-mediated mechanisms. Melatonin derived from the pineal gland and eyes therefore may act as an inhibitory factor of 7α-hydroxy pregnenolone biosynthesis in the quail. This notion is supported by earlier studies indicating that melatonin treatment decreases locomotor activity in quail (Murakami et al., 2001; Nakahara et al., 2003) and other birds (Murakami et al., 2001). To the best of our knowledge, this is the first observation showing that melatonin regulates neurosteroid biosynthesis in the brain of vertebrates (for a review, see Tsutsui et al., 2009b).
In quail, as in all vertebrates, the nocturnal secretion of melatonin is night-length dependent (Cockrem and Follett, 1983), and the onset of melatonin secretion occurs soon after the onset of darkness (Kumar and Follett, 1993). Therefore, the increase in 7α-hydroxyprogrenolone biosynthesis in the brain of male quail during the light period is likely to be a result of the decrease in endogenous melatonin secretion. Since 7α-hydroxyprogrenolone stimulates locomotor activity, it is proposed that, in male quail, this neurosteroid plays a crucial role in diurnal changes in locomotor activity through the action of melatonin.

In birds and vertebrates in general, locomotor activity undergoes a circadian rhythm (Saper et al., 2005) controlled by diurnal rhythm of melatonin secretion (Binkley et al., 1971; John et al., 1978; Cassone and Menaker, 1984; Chabot and Menaker, 1992; Hau and Gwinner, 1994; Warren and Cassone, 1995). However, the molecular mechanisms underlying this neurohormonal regulation of behavior are poorly understood. The discovery of the role of 7α-hydroxyprogrenolone in mediating the action of melatonin on diurnal locomotor rhythmicity is an important step in understanding these mechanisms (Tsutsui et al., 2008). A similar mechanism may underlie the regulation of diurnal locomotor rhythms in other vertebrates (for reviews, see Tsutsui et al., 2009a,b, 2010), since 7α-hydroxyprogrenolone is also present in the brains of newts (Matsunaga et al., 2004) and mammals (Akva et al., 1992; Dootzadeh and Morfin, 1997; Weill-Engerer et al., 2003; Yau et al., 2003).

SEASONAL CHANGES IN 7α-HYDROXYPROGENOLONE SYNTHESIS AND LOCOMOTOR ACTIVITY AND THEIR REGULATORY MECHANISMS

To understand the functional significance of 7α-hydroxyprogrenolone, seasonal changes in 7α-hydroxyprogrenolone biosynthesis and concentration in the brain were also demonstrated in the newt C. pyrrhogaster, a seasonally breeding wild animal (Matsunaga et al., 2004; Haraguchi et al., 2010). Seasonally breeding wild animals are suitable models to investigate such changes in neurosteroid production. Both the biosynthesis and concentration of 7α-hydroxyprogrenolone in the male brain markedly changed during the annual breeding cycle and were maximum in the spring breeding period (Figures 7A,B; Matsunaga et al., 2004; Haraguchi et al., 2009, 2010). Similar seasonal changes in the expression of newt P450ααα, that catalyzes the formation of 7α-hydroxyprogrenolone, occurred in the male brain (Figure 7C; Haraguchi et al., 2010). As mentioned in Section “Sex Differences in 7α-Hydroxyprogrenolone Synthesis and Locomotor Activity,” it has been previously reported that sexually mature male newts in the spring breeding period move around much more than females (Tsutsui, 1931; Iwata et al., 2000). These findings suggest that the increase in locomotor activity of male newts in the spring breeding period can be accounted for an increase in 7α-hydroxyprogrenolone biosynthesis in the brain. In contrast to males, 7α-hydroxyprogrenolone levels in the brain of females did not vary significantly and are constantly low (Figures 7A–C; Haraguchi et al., 2010). Accordingly, the lower locomotor activity in females could be ascribed to a lower level of 7α-hydroxyprogrenolone in their brain.

We have examined the mechanism that regulates seasonal changes in 7α-hydroxyprogrenolone biosynthesis in the male brain. In the newt, prolactin (PRL) induces development of sex characters (Dent, 1975); migration to water, in which sperm transfer and oviposition take place (Chadwick, 1941); development of the abdominal gland of the cloaca (Kikuyama et al., 1975), which secretes female-attracting pheromones (Kikuyama et al., 1995); enlargement of Mauthner neurons, which facilitate the rapid tail-vibration performed by male newts during courtship (Matsumoto et al., 1995); and expression of courtship behavior (Toyoda et al., 1993). Plasma PRL levels in the newt are elevated during the breeding period (Matsumoto et al., 1990; Mosconi et al., 1994) and it has been shown that PRL acts directly on the brain to regulate courtship behavior in the male newt (Toyoda et al., 2005). Based on these observations, we hypothesized that PRL may act on the brain to increase 7α-hydroxyprogrenolone biosynthesis, thus enhancing locomotor activity of male newts during the breeding period. A recent study has provided evidence that PRL is an important regulator of 7α-hydroxyprogrenolone production (Haraguchi et al., 2010). Hypophysectomy (Hypox) decreased after 2 weeks 7α-hydroxyprogrenolone biosynthesis and concentration in the brain of sexually mature males (Figure 8), suggesting that some pituitary hormone(s) may be involved in the regulation of 7α-hydroxyprogrenolone biosynthesis in the

FIGURE 6 | Effects of pinealectomy combined with orbital enucleation (Px + Ex) and melatonin administration to Px + Ex quail (Px + Ex + Mel) on 7α-hydroxyprogrenolone concentration. 7α- and 7β-hydroxyprogrenolone synthesis, and P450ααα mRNA expression in the diencephalon of male quail. (A) 7α-hydroxyprogrenolone concentration in the diencephalon. (B) 7α- and 7β-hydroxyprogrenolone synthesis in the diencephalon. (C) P450ααα mRNA expression in the diencephalon. Each column and vertical line represents the mean ± SEM (n = 5). **p < 0.01 vs. sham-operation (SH); *p < 0.01 Px + Ex vs. Px + Ex + Mel by one-way ANOVA, followed by Duncan’s multiple range test. Adapted from Tsutsui et al. (2008).
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Haraguchi et al., 2010). Administration of PRL but not gonadotropins (GTHs) to Hypox male newts caused a dose-dependent increase in α-hydroxypregnenolone biosynthesis (Figures 8A,B) and concentration in the brain (Haraguchi et al., 2010). Reciprocally, administration of anti-newt PRL serum dose-dependently decreased α-hydroxypregnenolone biosynthesis (Haraguchi et al., 2010). Accordingly, PRL secreted by the adenohypophysis can be regarded as a major factor regulating α-hydroxypregnenolone biosynthesis. This is a previously undescribed role of the adenohypophyseal hormone in the regulation of neurosteroidogenesis in the brain in any vertebrate. Further studies are needed to clarify whether a similar hormonal mechanism regulating α-hydroxypregnenolone biosynthesis occurs in other vertebrates.

In contrast to male newts, no seasonal changes in α-hydroxypregnenolone biosynthesis and concentration, and P450α mRNA expression were observed in female newts (Figure 7; Haraguchi et al., 2010). Interestingly, plasma PRL levels in the male newt C. pyrrhogaster exhibit marked seasonal changes during the annual breeding cycle and are maximum in the spring breeding period. In contrast, plasma PRL levels in females are constantly low (Matsuda et al., 1990). Such a sex difference in the seasonal changes in plasma PRL levels may account for the absence of seasonal changes in α-hydroxypregnenolone biosynthesis and concentration, and P450α mRNA expression in the female brain.

To understand the mode of action of PRL in the regulation of α-hydroxypregnenolone biosynthesis, we have determined the site of expression of P450α and looked for colocalization of P450α mRNA and PRL receptor (PRLR) in sexually mature male newts. P450α mRNA-positive cells were localized mainly in the anterior preoptic area (POA), magnocellular preoptic nucleus (Mg), and tegmental area (TA) in the brain (Haraguchi et al., 2010). However, PRLR-like immunoreactivity was found only in the Mg (Haraguchi et al., 2010). Thus, the major, but perhaps not exclusive, targets of PRL action to increase α-hydroxypregnenolone biosynthesis are the P450α-positive cells in the Mg (Figure 9). The Mg is sexually dimorphic both in term of response to pheromones and neuroanatomical aspect (Govek and Swann, 2007). In particular, the Mg possesses more neurons in the male than in the female (Govek et al., 2003). Electrolytic lesions that include the Mg immediately and permanently eliminate male copulatory behavior in the hamster (Powers et al., 1987). In newt (Giorgio et al., 1982; Toyoda et al., 1993), the involvement of PRL in eliciting courtship behavior of males has been reported. Accordingly, it is possible that PRL may also induce the expression of courtship behavior by increasing α-hydroxypregnenolone synthesis in the Mg of sexually mature male newts. In addition, it has been reported that PRL acts on the Mg to cause the release of arginine vasotocin (AVT; Hasunuma et al., 2007; Kikuyama et al., 2009). AVT is known to be an important factor for the expression of courtship behavior (Toyoda et al., 2003). Therefore, it is possible that PRL-induced courtship behavior in male newt is mediated by AVT release, which may act to stimulate biosynthesis of α-hydroxypregnenolone.

On the other hand, it is known that, in mammals, PRL is synthesized in not only the adenohypophysis but also a subset of hypothalamic neurons projecting throughout the brain.
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and function of newt brain PRL are still unclear. It is considered that adenohypophysis PRL is more important than brain PRL in the expressions of locomotor activity and courtship behavior, inasmuch as an increase in plasma PRL levels in breeding male newts (Matsuda et al., 1990; Mosconi et al., 1994) and the suppression of courtship behavior in Hypox male newts (Toyoda et al., 1993) have also been reported. In the choroid plexus of newt, dense PRLR immunoreactivity and PRLR mRNA signals were observed the epithelial cells (Hasunuma et al., 2005). In mammals, choroid plexus PRLR has been proposed to be involved in the transport of PRL from blood into the cerebrospinal fluid (Walsh et al., 1987). Thus, PRL transported from the blood into the cerebrospinal fluid via the choroid plexus receptor is considered to play an important role in the expression of courtship behavior, although a possible contribution of PRL transported to the brain through retrograde blood flow by the portal system as reported in mammals (Oliver et al., 1977; Porter et al., 1978) cannot be excluded.

**CONCLUSION**

The brain of vertebrates possesses several kinds of steroidogenic enzymes and produces a variety of neurosteroids. However, the biosynthetic pathway of neurosteroids in the brain was not completely elucidated. A newly discovered amphibian and avian neurosteroid, 7α-hydroxyprogrenolone, acts as an important factor stimulating locomotor activity. The stimulatory action of 7α-hydroxyprogrenolone is mediated by the dopaminergic system. 7α-Hydroxyprogrenolone apparently functions in male but not in female. Melatonin acts on the neurons expressing P450\textsubscript{7α} to regulate 7α-hydroxyprogrenolone biosynthesis, thus inducing diurnal locomotor changes in males. PRL, an adenohypophyseal hormone, also acts on the neurons expressing P450\textsubscript{7α} to regulate 7α-hydroxyprogrenolone biosynthesis, thus inducing seasonal

( Fukue et al., 1977; De Vito, 1988; Emanuele et al., 1992). In preliminary experiments, we performed RT-PCR using newt brain total RNA with newt PRL cDNA-specific primers and detected specific amplification (unpublished data). In contrast, by an immunohistochemical method using anti-newt PRL antiserum, we could not detect PRL immunoreactivity in the newt brain. These results suggest that PRL is expressed in the newt brain but the expression level might be very low. Thus, the localization

![Figure 8](image1.png)

**FIGURE 8** Effects of hypophysectomy (Hypox) and administration of PRL or GTH on 7α-hydroxyprogrenolone synthesis and concentration in the brain of breeding male newts. (A) Effects of Hypox and i.c.v. injection of various doses of PRL on 7α-hydroxyprogrenolone synthesis. (B) Effects of Hypox and i.c.v. injection of various doses of GTH on 7α-hydroxyprogrenolone synthesis. Each column and vertical line represents the mean ± SEM (n = 6). *p < 0.05 vs. sham-operation (SH) plus saline; †p < 0.05 vs. Hypox plus saline by one-way ANOVA, followed by Tukey–Kramer test. Adapted from Haraguchi et al. (2010).

![Figure 9](image2.png)

**FIGURE 9** Schematic model depicting the possible action of PRL on the expression of P450\textsubscript{7α} in the magnocellular preoptic nucleus (Mg) of male newt. PRL synthesized in the adenohypophysis, by acting on Mg neurons in the hypothalamus, may induce the expression of P450\textsubscript{7α}. P450\textsubscript{7α} and PRL receptor (PRLR) are colocalized in Mg neurons. Thus, the major, but perhaps not exclusive, targets of PRL action to increase 7α-hydroxyprogrenolone biosynthesis are Mg neurons expressing P450\textsubscript{7α}. See the text for details.
locomotor changes in males. These recent findings indicate that 7α-hydroxypregnenolone-producing neurons may play a pivotal role in the integration of circadian and seasonal information that affects locomotor activity in amphibians and birds.

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