frontiers in
ENDOCRINOLOGY

REVIEW ARTICLE
published: 21 September 2011
doi: 10.3389/fendo.2011.00037

Neurosteroid biosynthesis and function in the brain of domestic birds

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It is now established that the brain and other nervous systems have the capability of forming steroids *de novo*, the so-called "neurosteroids." The pioneering discovery of Baulieu and his colleagues, using rodents, has opened the door to a new research field of "neurosteroids." In contrast to mammalian vertebrates, little has been known regarding *de novo* neurosteroidogenesis in the brain of birds. We therefore investigated neurosteroid formation and metabolism in the brain of quail, a domestic bird. Our studies over the past two decades demonstrated that the quail brain possesses cytochrome P450 side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD), 5β-reductase, cytochrome P450 17α-hydroxylase/c17,20-lyase ($P450_{17\alpha,lyase}$), 17β-HSD, etc., and produces pregnenolone, progesterone, 5β-dihydroprogesterone (5β-DHP), 3β, 5β-tetrahydroprogesterone (3β, 5β-THP), androstenedione, testosterone, and estradiol from cholesterol. Independently, Schlinger's laboratory demonstrated that the brain of zebra finch, a songbird, also produces various neurosteroids. Thus, the formation and metabolism of neurosteroids from cholesterol is now known to occur in the brain of birds. In addition, we recently found that the quail brain expresses cytochrome $P450_{7\alpha}$ and produces 7α- and 7β-hydroxypregnenolone, previously undescribed avian neurosteroids, from pregnenolone. This paper summarizes the advances made in our understanding of neurosteroid formation and metabolism in the brain of domestic birds. This paper also describes what are currently known about physiological changes in neurosteroid formation and biological functions of neurosteroids in the brain of domestic and other birds.

Keywords: neurosteroids, neurosteroidogenesis, steroidogenic enzymes, neurosteroid function, brain, quail, domestic birds

INTRODUCTION

Peripheral steroid hormones cross the blood–brain barriers and act on brain cells through intracellular receptor-mediated mechanisms that regulate several important brain neuronal functions. Therefore, the brain traditionally has been considered to be a target site of peripheral steroid hormones. In contrast to this classical concept, new findings over the past two decades have shown that the brain itself also synthesizes steroids *de novo* from cholesterol through mechanisms at least partly independent of peripheral steroidogenic glands. Such steroids synthesized *de novo* in the brain and other nervous systems are called "neurosteroids." Baulieu and colleagues have opened the door to a new research field of "neurosteroids" from their studies using rodents. In contrast to mammalian vertebrates, little has been known regarding *de novo* neurosteroidogenesis in the brain of birds.

Birds have always served as excellent animal models for understanding the actions of peripheral steroids on brain and behavior. Thus, the investigation of neurosteroid synthesis and action in birds may be also useful. We therefore characterized neurosteroids formed from cholesterol in the avian brain using the Japanese quail *Coturnix japonica*. A series of our studies over the past two decades demonstrated that the quail brain possesses cytochrome P450 side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-isomerase (3β-HSD), 5β-reductase, cytochrome P450 17α-hydroxylase/c17,20-lyase ($P450_{17\alpha,lyase}$), 17β-HSD, etc., and produces pregnenolone, progesterone, 5β-dihydroprogesterone (5β-DHP), 3β, 5β-tetrahydroprogesterone (3β, 5β-THP), androstenedione, testosterone, and estradiol from cholesterol (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Tsutsui et al., 1997a,b, 1999, 2003a,b; Ukena et al., 1999a,b, 2001; Matsunaga et al., 2001, 2002; Tsutsui and Schlinger, 2001). Schlinger and colleagues also undertook similar studies in the zebra finch *Taeniopygia guttata* (Vanson et al., 1996; Schlinger et al., 1999; Freking et al., 2000; Soma et al., 2004). Thus, the formation and metabolism of several neurosteroids from cholesterol appear to occur in the brain of birds.

In addition to mammals and birds, the formation and metabolism of neurosteroids from cholesterol is now well documented in amphibians (Mensah-Nyagan et al., 1994, 1996a,b, 1999; Beaujean 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 is considered to be a conserved property across vertebrates (for reviews, see Baulieu, 1997; Tsutsui et al., 1999, 2000, 2003a,b, 2006; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Tsutsui and Mellon,

2006; Do-Rego et al., 2009). However, biosynthetic pathways of neurosteroid in birds, as well as other vertebrates may still not be fully mapped (for a review, see Tsutsui et al., 2006). This conclusion is supported by the discovery that the quail brain synthesizes two previously undescribed avian neurosteroids, 7α- and 7β-hydroxypregnenolone, from pregnenolone (Tsutsui et al., 2008). Furthermore, cytochrome $P450_{7\alpha}$ catalyzing the conversion of pregnenolone to 7α-hydroxypregnenolone was identified in the quail brain (Tsutsui et al., 2008).

This review summarizes neurosteroid formation and metabolism in the brain of domestic birds, in particular the quail, obtained by a series of our studies and the related studies with the ring dove (Clark et al., 1999; Tsutsui et al., 1999; Lea et al., 2001), by the collaboration with the laboratory of Lea at the University of Central Lancashire, Preston, UK. Based on recent findings, this review also highlights the current knowledge regarding physiological changes in neurosteroid formation and biological functions of neurosteroids in the avian brain.

BACKGROUND OF THE DISCOVERY OF NEUROSTEROIDS IN THE AVIAN BRAIN

A great deal was known about the brain as a target site of peripheral steroid hormones more than 20 years ago. 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., 1977; McEwen, 1991). By diverse actions on the brain, peripheral steroids, in particular sex steroids, have profound effects on behavior of vertebrate animals. Birds have contributed significantly to our understanding of the mechanisms of steroid actions on several kinds of reproductive behaviors, such as courtship, copulatory, aggressive, and parental behaviors. Extensive studies using a variety of wild and captive, intact and castrated, reproductive and non-reproductive birds have established a relationship between the blood level of androgens, estrogens, or progestins and the expression of typical reproductive behaviors (Ottinger and Brinkley, 1978, 1979; Tsutsui and Ishii, 1981; Balthazart, 1983; Wingfield and Marler, 1988; Schlinger and Callard, 1991; Wingfield and Farner, 1993).

Gonadal androgens, for instance, act on the brain to influence several male reproductive behaviors in birds. 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). Many of the brain regions that control a variety of reproductive behaviors contain 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 traditionally has been considered to be a target site of peripheral steroids. In addition to direct steroid actions, the metabolism of peripheral steroids in brain tissues can result in biotransformation and the production of biologically active metabolites. Androgenic action in the brain is often mediated by the enzymatic activity of cytochrome P450 aromatase (P450arom) which catalyzes the conversion of androgens to estrogens. Both cytochrome P450arom and estrogen receptors are expressed in several brain regions including the hypothalamus and preoptic area which are involved in the control of reproductive behaviors in birds (Schlinger and Callard, 1987, 1989a,b,c, 1991; Balthazart et al., 1990a,b, 1991).

On the other hand, new findings from several laboratories have established unequivocally that the nervous system itself forms neurosteroids *de novo* from cholesterol. This new concept originated from the observations made by Baulieu and colleagues. They found that several neurosteroids, such as pregnenolone, dehydroepiandrosterone, and their esters, accumulate in high quantities in 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 neurosteroids remain constant even after the removal of peripheral steroids by procedures, such as adrenalectomy, castration, and hypophysectomy. Extensive studies on mammals have established that the brain and other nervous systems have the capability of forming neurosteroids *de novo* from cholesterol (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Robel et al., 1986, 1987; Jo et al., 1989).

In birds, the new concept of *de novo* neurosteroidogenesis in the brain originated from our studies in the 1990s. We demonstrated that various neurosteroids are formed from cholesterol in the brain of quail (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Tsutsui et al., 1997; Tsutsui et al., 1999, 2003a; Ukena et al., 1999; Ukena et al., 2001; Matsunaga et al., 2001, 2002; Tsutsui and Schlinger, 2001). Independently, Schlinger's laboratory at the University of California, Los Angeles, USA also contributed to this area by the studies using zebra finches (Vanson et al., 1996; Schlinger et al., 1999; Freking et al., 2000; Soma et al., 2004). The formation and metabolism of neurosteroids is now known to occur in the brain of birds in general.

EXPRESSION OF CYTOCHROME P450SCC AND PREGNENOLONE FORMATION IN THE AVIAN BRAIN

Pregnenolone, a 3β-hydroxy-Δ^5-steroid, is known to be the common precursor of all steroid hormones in peripheral steroidogenic glands including the gonads and adrenals (**Figure 1**). 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 (**Figure 1**). Therefore, the demonstration of pregnenolone formation from cholesterol is essential to establish *de novo* neurosteroidogenesis in the brain of birds.

EXPRESSION AND ENZYMATIC ACTIVITY OF CYTOCHROME P450SCC IN THE AVIAN BRAIN

Tsutsui and Yamazaki (1995) measured the concentration of pregnenolone in the brain of adult quail using a specific radioimmunoassay. The pregnenolone concentration in adult birds was much higher in the brain than in plasma (Tsutsui and Yamazaki, 1995). 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 (Tsutsui and Yamazaki, 1995). Subsequently, the formation of pregnenolone from cholesterol was found in intact

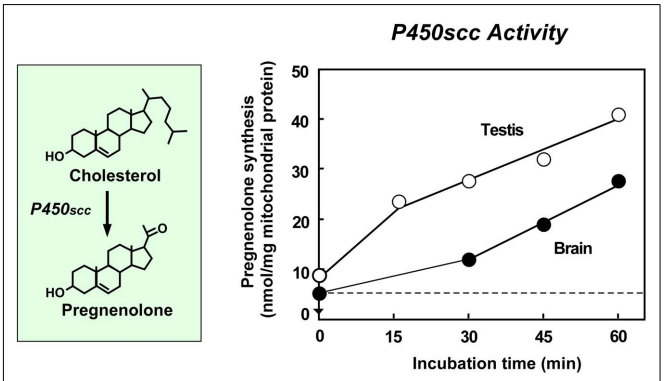

FIGURE 1 | Pregnenolone formation from cholesterol in the quail brain. Biochemical analysis of the formation of pregnenolone from cholesterol using intact mitochondria derived from the quail brain. Incubations were performed with 200 μM cholesterol, 0.25 mg of mitochondrial protein, 10 mM isocitrate and 10 mM succinate at 41°C for different times. See Tsutsui and Yamazaki (1995) for details.

mitochondria derived from the quail brain (Tsutsui and Yamazaki, 1995; **Figure 1**). 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 the ring dove, another domestic bird (Clark et al., 1999; Tsutsui et al., 1999; Lea et al., 2001), and the zebra finch, a songbird (Freking et al., 2000). 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,b, 1999; Tsutsui and Schlinger, 2001; Tsutsui et al., 2003a).

The presence of cytochrome P450scc and pregnenolone formation in the brain are considered as a conserved property of vertebrates, because amphibians also possess cytochrome P450scc in the brain (Takase et al., 1999; Inai et al., 2003), like birds (Tsutsui and Yamazaki, 1995; Tsutsui et al., 1997a,b, 1999; Clark et al., 1999; Freking et al., 2000; Lea et al., 2001) and mammals (Corpéchot 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; Jung-Testas et al., 1989; Jo 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).

LOCALIZATION OF CYTOCHROME P450SCC IN THE AVIAN BRAIN

To understand the formation of pregnenolone in the avian brain, we need to clarify the localization of steroidogenic cells expressing cytochrome P450scc in the brain. Immunohistochemical studies of the quail brain using antiserum against cytochrome P450scc (Usui et al., 1995; Tsutsui et al., 1997a) indicated that clusters of immunoreactive cells are detected in the hyperstriatum accessorium, the ventral portions of the archistriatum and the corticoid area, the preoptic area, the anterior hypothalamus, and the dorsolateral thalamus. Western immunoblot analysis confirmed the presence of cytochrome P450scc in the immunohistochemically identified brain regions (Usui et al., 1995; Tsutsui et al., 1997a). In mammals, glial cells were first accepted to play a role in neurosteroid formation and metabolism in the brain. 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 may be also true for the presence of cytochrome 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).

The concept of *de novo* neurosteroidogenesis in neurons in the brain had been uncertain in all vertebrates. In the middle 1990s, however we found that the Purkinje cell, a cerebellar neuron, possesses cytochrome P450scc, and produces pregnenolone from cholesterol (Usui et al., 1995; Tsutsui et al., 1997a). In immunohistochemical studies, the striking observation in the quail brain was the distribution of immunoreactive cells in the cerebellar cortex. The distribution of immunoreactive cell bodies and fibers in the cerebellar cortex coincided with the location of somata and dendrites of Purkinje cells (Usui et al., 1995; Tsutsui et al., 1997a). Western immunoblot analysis also confirmed the presence of cytochrome P450scc in this neuron (Usui et al., 1995; Tsutsui et al., 1997a). These findings in birds have provided the first evidence for the location of cytochrome P450scc in the neuron in the brain. Our biochemical and molecular studies on mammals and amphibians further identified the presence of cytochrome P450scc in the Purkinje cell (Ukena et al., 1998, 1999b; Takase et al., 1999; Tsutsui and Ukena, 1999; Tsutsui et al., 2000, 2003b; Inai et al., 2003).

EXPRESSION OF CYTOCHROME $P450_{7\alpha}$ AND FORMATION OF 7α- AND 7β-HYDROXYPREGNENOLONE IN THE AVIAN BRAIN

As mentioned above, pregnenolone formation from cholesterol in the brain has been documented in birds (**Figure 1**). In the avian brain, we recently identified 7α- and 7β-hydroxypregnenolone as novel pregnenolone metabolites that are not known to be precursors of progestins, androgens, or estrogens (Tsutsui et al., 2008; **Figure 2**). Subsequently, we demonstrated that 7α-hydroxypregnenolone is converted from pregnenolone through the enzymatic activity of cytochrome $P450_{7\alpha}$ (Tsutsui et al., 2008; **Figure 2**).

IDENTIFICATION, EXPRESSION, AND ENZYMATIC ACTIVITY OF CYTOCHROME $P450_{7\alpha}$ IN THE AVIAN BRAIN

We initially found that the quail brain actively produces unknown neurosteroids from pregnenolone. We therefore sought to identify these avian neurosteroids from the brain of adult quail by using biochemical techniques combined with high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and gas chromatography–mass spectrometry (GC–MS) analyses (Tsutsui et al., 2008). Quail brain homogenates were incubated with tritiated pregnenolone as a precursor, and radioactive metabolites were analyzed by reversed-phase HPLC. Several non-radioactive steroids were used as reference standards for HPLC analysis, and 7α-hydroxypregnenolone and its stereoisomer 7β-hydroxypregnenolone exhibited the same retention time of

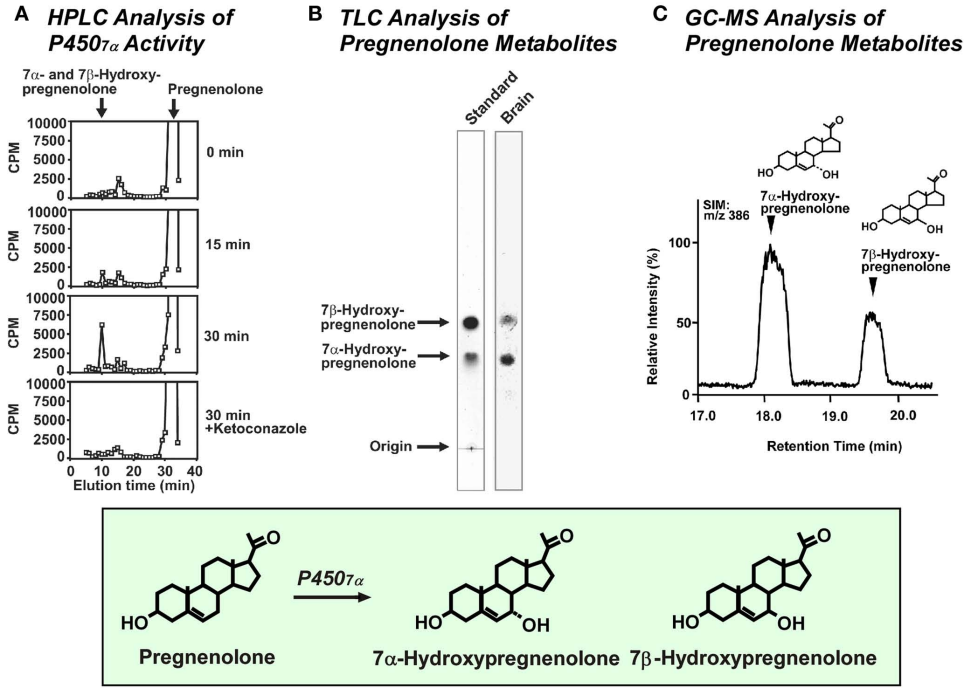

FIGURE 2 | Conversion of pregnenolone to 7α- and 7β-hydroxypregnenolone in the quail brain. (A) HPLC profile of unknown metabolites of pregnenolone by using a reversed-phase column. Quail brain homogenates were incubated with ^{3}H-pregnenolone, and the extracts were subjected to HPLC. The ordinate indicates the radioactivity measured in each HPLC fraction, and the arrows indicate elution positions of standard steroids, pregnenolone and 7α- and 7β-hydroxypregnenolone. **(B)** Autoradiography of the unknown pregnenolone metabolites (right column) and standard steroids 7α- and 7β-hydroxypregnenolone (left column) on TLC under the same condition as in **(A)**. **(C)** GC–selected ion monitoring (SIM) mass trace of *m/z* 386 in the extract from quail brain homogenates. The arrowheads indicate the retention times of 7α-hydroxypregnenolone and 7β-hydroxypregnenolone. See Tsutsui et al. (2008) for details.

the radioactive peak under a similar chromatographic condition (Tsutsui et al., 2008; **Figure 2A**). The detection of ^{3}H-7α- and 7β-hydroxypregnenolone was feasible by HPLC because the radioactive pregnenolone was labeled with ^{3}H at multiple positions, including the 7α- or 7β-position. The HPLC peak was collected and subjected to TLC. Quail brain homogenates produced two metabolites from ^{3}H-pregnenolone corresponding to the positions of the 7α- and 7β-hydroxypregnenolone standards (Tsutsui et al., 2008; **Figure 2B**). The metabolites of pregnenolone were further analyzed by GC–MS. Based on GC–selected ion monitoring (SIM) analysis (*m/z* 386), the metabolites had retention times that were identical to 7α-hydroxypregnenolone and 7β-hydroxypregnenolone (Tsutsui et al., 2008; **Figure 2C**).

7α-Hydroxypregnenolone is synthesized from pregnenolone through the enzymatic activity of cytochrome P450$_{7\alpha}$ (**Figure 2**). In order to prove that 7α-hydroxypregnenolone is synthesized in the brain, it is therefore necessary to show that brain expresses P450$_{7\alpha}$. A full length, 2341 bp cDNA prepared from the quail brain tissue was identified to encode a putative cytochrome P450$_{7\alpha}$ (Tsutsui et al., 2008). The putative quail P450$_{7\alpha}$ 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 P450$_{7\alpha}$ was demonstrated using homogenates of COS-7 cells transfected with the putative quail P450$_{7\alpha}$ cDNA (Tsutsui et al., 2008). HPLC analyses demonstrated that the homogenate converts pregnenolone to 7α- and/or 7β-hydroxypregnenolone (Tsutsui et al., 2008). Subsequently, 7α-hydroxypregnenolone but not 7β-hydroxypregnenolone synthesis was confirmed by GC–MS (Tsutsui et al., 2008). Although it is still unclear whether cytochrome P450$_{7\alpha}$ can also convert pregnenolone to 7β-hydroxypregnenolone, the presence of 7β-hydroxypregnenolone as well as 7α-hydroxypregnenolone is evident in the quail brain (Tsutsui et al., 2008; **Figure 2**).

The production of 7α-hydroxypregnenolone in the brain may be a conserved property of vertebrates because this neurosteroid has also been identified in the brain 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). Recently, a cDNA encoding cytochrome P450$_{7\alpha}$ was identified in the newt brain tissue (Haraguchi et al., 2010). The homogenate of COS-7 cells transfected with the newt P450$_{7\alpha}$ cDNA also converted pregnenolone into 7α-hydroxypregnenolone (Haraguchi et al., 2010).

LOCALIZATION OF CYTOCHROME P450$_{7\alpha}$ IN THE AVIAN BRAIN

To understand the localization of cytochrome P450$_{7\alpha}$ in the avian brain, the biosynthesis and concentrations of 7α- and 7β-hydroxypregnenolone were compared in different regions of the quail brain in both sexes by HPLC and GC–MS analyses, respectively (Tsutsui et al., 2008). The two neurosteroids were

found predominantly in the diencephalon and were very low in other brain regions (Tsutsui et al., 2008). The biosynthesis and concentrations of 7α- and 7β-hydroxypregnenolone in the diencephalon were found to be sexually differentiated being much higher in males than in females (Tsutsui et al., 2008). Such a sexual dimorphism of cytochrome $P450_{7\alpha}$ only occurs in the diencephalon (Tsutsui et al., 2008). There are similar sex differences in 3β-HSD and cytochrome P450arom in the avian brain (Schlinger and Callard, 1987; Soma et al., 2004; Tam and Schlinger, 2007).

Tsutsui et al. (2008) further investigated the expression of cytochrome $P450_{7\alpha}$ by *in situ* hybridization to identify the cells producing 7α-hydroxypregnenolone in the quail brain. In the male diencephalon, the expression of cytochrome $P450_{7\alpha}$ mRNA was localized in the nucleus preopticus medialis, the nucleus paraventricularis magnocellularis, the nucleus ventromedialis hypothalami, the nucleus dorsolateralis anterior thalami and the nucleus lateralis anterior thalami (Tsutsui et al., 2008).

EXPRESSION OF 3β-HSD AND PROGESTERONE FORMATION IN THE AVIAN BRAIN

The biosynthesis of progesterone is performed by 3β-HSD that catalyzes oxidation and isomerization of Δ^5-3β-hydroxysteroids (pregnenolone and dehydroepiandrosterone) into Δ^4-ketosteroids (progesterone and androstenedione, respectively). 3β-HSD is highly expressed in the peripheral steroidogenic glands (Mason, 1993; **Figure 3**). Thus, the demonstration of 3β-HSD expression and progesterone production is essential in order to understand the biosynthetic pathway of neurosteroids in the avian brain.

EXPRESSION AND ENZYMATIC ACTIVITY OF 3β-HSD IN THE AVIAN BRAIN

Our biochemical and molecular studies have demonstrated the expression of 3β-HSD and the formation of progesterone in the brain of adult quail. RT-PCR analysis together with Southern hybridization showed the expression of 3β-HSD mRNA in the quail brain in both sexes (Ukena et al., 1999a; **Figure 3A**). By using biochemical techniques combined with HPLC analysis, Ukena et al. (1999a) demonstrated that, in the quail brain, pregnenolone is converted to progesterone (**Figure 3B**). The biosynthesis of progesterone increased with time and was completely abolished by trilostane, an inhibitor of 3β-HSD (Ukena et al., 1999a; **Figure 3B**). These studies indicate that the quail brain expresses 3β-HSD and converts pregnenolone to progesterone (**Figure 3**).

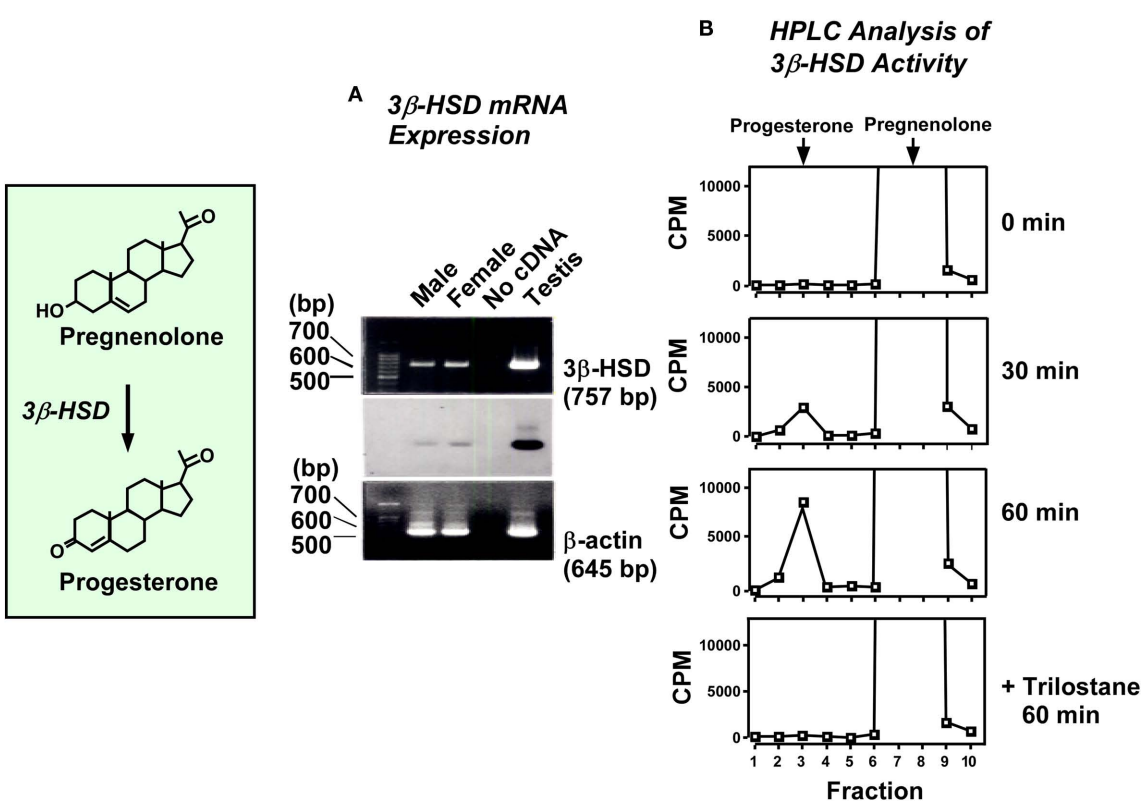

FIGURE 3 | Progesterone formation from pregnenolone in the quail brain. **(A)** RT-PCR analysis of 3β-HSD mRNA in the quail brain. Upper panel shows a result of the gel electrophoresis of PT-PCR products for chicken 3β-HSD, and middle panel shows an identification of the band by Southern hybridization using digoxigenin-labeled oligonucleotide probe for chicken 3β-HSD. The lane labeled "No cDNA" was performed without template as the negative control. Lower panel shows a result of the RT-PCR for chicken β-actin as the internal control. **(B)** HPLC analysis of neurosteroids extracted from quail brain slices after different incubation times with ^{3}H-pregnenolone using a reversed-phase column. The column was eluted with a linear gradient of 40–70% acetonitrile. The ordinate indicates the radioactivity measured in each HPLC fraction. The arrows indicate the elution positions of pregnenolone and progesterone. The brain slices were also incubated with trilostane, a specific inhibitor of 3β-HSD, for 60 min and subjected to HPLC analysis. See Ukena et al. (1999a) for details.

The expression of 3β-HSD and its enzymatic activity were also found in the brain of zebra finches (Vanson et al., 1996) and ring doves (Lea et al., 2001). Thus, the avian brain possesses not only cytochrome P450scc but also 3β-HSD and produces progesterone *de novo* from cholesterol. The expression of both 3β-HSD protein and its mRNA has also been reported in mammalian brains (Dupont et al., 1994; Guennoun et al., 1995; Sanne and Krueger, 1995; Kohchi et al., 1998; Ukena et al., 1999b). 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., 1999b), amphibians (Mensah-Nyagan et al., 1994), and fish (Sakamoto et al., 2001).

LOCALIZATION OF 3β-HSD IN THE AVIAN BRAIN

Based on the biochemical analysis, we further analyzed the activity of 3β-HSD in all brain regions of the adult quail. The enzymatic activity in the telencephalon and diencephalon was higher than that in the mesencephalon (Ukena et al., 1999a). The progesterone level was also high in the telencephalon and diencephalon and low in the mesencephalon (Ukena et al., 1999a). It has been reported that rat 3β-HSD mRNA is expressed in several brain regions in particular, olfactory bulb, striatum, cortex, thalamus, hypothalamus, habenula, septum, hippocampus, and cerebellum (Guennoun et al., 1995). Although the exact site showing 3β-HSD expression is still obscure in the quail brain, the regional distribution of 3β-HSD reported in the rat brain correlates with that of cytochrome P450scc in the quail brain. Therefore, sites having both steroidogenic enzymes can synthesize progesterone *de novo* from cholesterol.

EXPRESSION OF 5β-REDUCTASE AND PROGESTERONE METABOLISM IN THE AVIAN BRAIN

To understand the pathway of progesterone metabolism, we investigated progesterone metabolites in the brain of adult quail. Biochemical analysis together with HPLC and TLC revealed that the quail brain produces 5β-DHP from progesterone (Ukena et al., 2001; **Figure 4**). We further demonstrated that the quail brain produces not only 5β-DHP but also 3β, 5β-THP from progesterone (Tsutsui et al., 2003a; **Figure 4**). In birds, 5β-reduction also represents a route of androgen metabolism in the brain (Massa and Sharp, 1981; Schlinger and Callard, 1987).

Thus, the progesterone metabolites, 5β-DHP and 3β, 5β-THP, are produced by the enzymatic activities of 5β-reductase and 3β-HSD, and accumulate in the avian brain as neurosteroids (**Figure 4**). In contrast to birds, progesterone is converted to 5α-DHP and 3α, 5α-THP due to 5α-reductase and 3α-HSD in mammals (for reviews, see Baulieu, 1997; Compagnone and Mellon, 2000).

EXPRESSIONS OF CYTOCHROME $P450_{17\alpha,LYASE}$ AND 17β-HSD AND ANDROGEN FORMATION IN THE AVIAN BRAIN

Another pathway of progesterone metabolism is mediated by cytochrome $P450_{17\alpha,lyase}$ which, in addition to converting pregnenolone to dehydroepiandrosterone via 17α-hydroxypregnenolone, also converts progesterone to androstenedione via 17α-hydroxyprogesterone (**Figures 5** and **8**). Both of these metabolic pathways was demonstrated in the quail brain

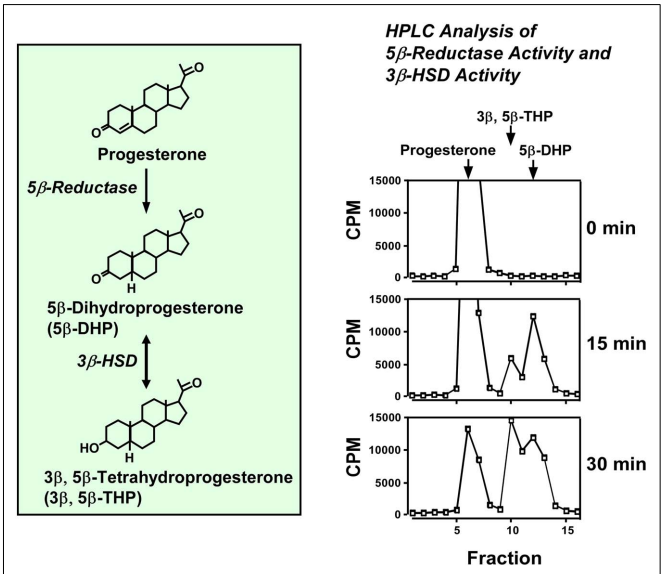

FIGURE 4 | Conversion of progesterone to 5β-DHP and 3β, 5β-THP in the quail brain. HPLC analysis of neurosteroids extracted from quail brain homogenates after different incubation times with ^{3}H-progesterone using a reversed-phase column. The column was eluted with an isocratic elution of 70% acetonitrile. The ordinate indicates the radioactivity measured in each HPLC fraction. The arrows indicate the elution positions of progesterone, 5β-DHP and 3β, 5β-THP. See Ukena et al. (2001) for details.

using biochemical techniques combined with HPLC analysis, and by RT-PCR analysis of cytochrome $P450_{17\alpha,lyase}$ mRNA (Matsunaga et al., 2001, 2002; **Figure 5**). We further demonstrated that the avian brain expresses 17β-HSD that is needed to convert androstenedione to testosterone (Matsunaga et al., 2002; **Figure 6**).

EXPRESSION AND ENZYMATIC ACTIVITY OF CYTOCHROME $P450_{17\alpha,LYASE}$ IN THE AVIAN BRAIN

In contrast to the presence of cytochrome P450scc, 3β-HSD, and 5β-reductase, limited information has been available for cytochrome $P450_{17\alpha,lyase}$ in the brain of birds as well as other vertebrates. We therefore investigated the expression of cytochrome $P450_{17\alpha,lyase}$ in the brain of adult quail (Matsunaga et al., 2001). RT-PCR analysis followed by Southern hybridization indicated the expression of cytochrome $P450_{17\alpha,lyase}$ mRNA in the quail brain in both sexes (Matsunaga et al., 2001; **Figure 5A**). Employing biochemical techniques combined with HPLC analysis, the conversion of progesterone to 17α-hydroxyprogesterone (Matsunaga et al., 2001) and subsequently to androstenedione (Matsunaga et al., 2002; **Figure 5B**) was also found in quail brain slices. Thus, it appears that the avian brain produces androstenedione from progesterone (**Figure 5**). The expression of cytochrome $P450_{17\alpha,lyase}$ in the brain was also detected in mammals (Compagnone et al., 1995; Strömstedt and Waterman, 1995; Kohchi et al., 1998). Therefore, the expression of cytochrome $P450_{17\alpha,lyase}$ in the brain is considered to be a conserved property of vertebrates.

LOCALIZATION OF CYTOCHROME $P450_{17\alpha,LYASE}$ IN THE AVIAN BRAIN

Birds showed clearly a region-dependent expression of cytochrome $P450_{17\alpha,lyase}$ in the brain (Matsunaga et al., 2001).

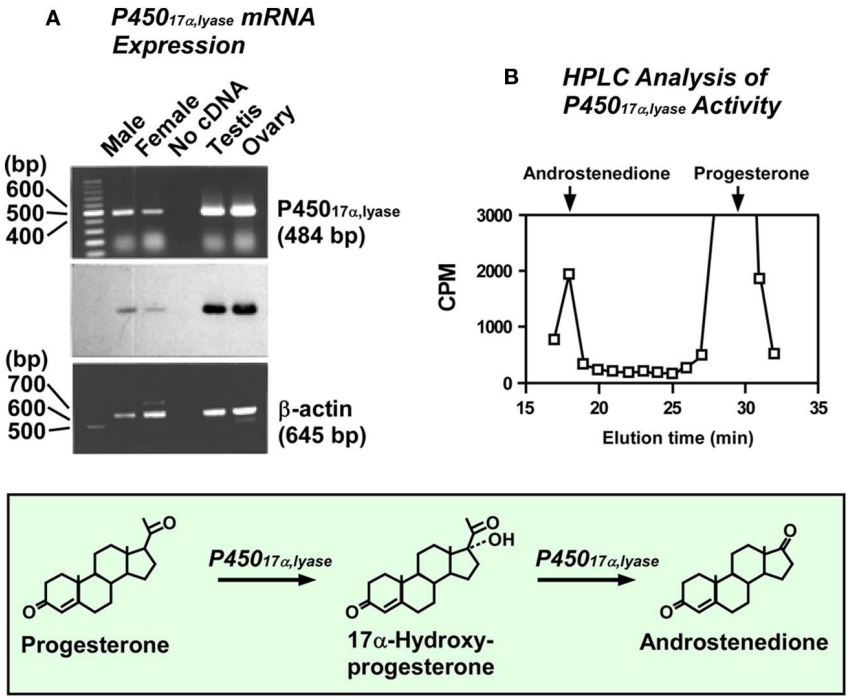

FIGURE 5 | Conversion of progesterone to androstenedione in the quail brain. (A) RT-PCR analysis of cytochrome $P450_{17\alpha,lyase}$ mRNA in the quail brain. Upper panel shows a result of the gel electrophoresis of RT-PCR products for chicken $P450_{17\alpha,lyase}$, and middle panel shows an identification of the band by Southern hybridization using digoxigenin-labeled oligonucleotide probe for chicken $P450_{17\alpha,lyase}$. The lane labeled "No cDNA" was performed without template as the negative control. Lower panel shows a result of the RT-PCR for chicken β-actin as the internal control. **(B)** HPLC analysis of neurosteroids extracted from quail diencephalic slices after 60 min incubation with ^{3}H-progesterone using a reversed-phase column. The column was eluted with a 30-min linear gradient of 40–70% acetonitrile, followed by an isocratic elution of 70% acetonitrile. The ordinate indicates the radioactivity measured in each HPLC fraction. The arrows indicate the elution positions of progesterone and androstenedione. See Matsunaga et al. (2001, 2002) for details.

Based on RT-PCR analysis together with Southern hybridization, cytochrome $P450_{17\alpha,lyase}$ mRNA was highly expressed in the quail diencephalon and mesencephalon (Matsunaga et al., 2001). This result is in agreement with the findings reported in mammals (Compagnone et al., 1995; Strömstedt and Waterman, 1995; Kohchi et al., 1998). According to Kohchi et al. (1998), the cytochrome $P450_{17\alpha,lyase}$ mRNA expression was high in the mesencephalon, but it was very weak in the cerebrum and cerebellum. Strömstedt and Waterman (1995) also found, using RT-PCR analysis followed by Southern blots, a higher expression of the cytochrome $P450_{17\alpha,lyase}$ mRNA in the brain stem of rats and mice. In addition, Compagnone et al. (1995) reported that the rat embryonic cells expressing cytochrome $P450_{17\alpha,lyase}$ are located in the mesencephalic region as well as the medulla and spinal cord.

We further characterized the site showing cytochrome $P450_{17\alpha,lyase}$ expression by *in situ* hybridization. In the quail brain, the expression of cytochrome $P450_{17\alpha,lyase}$ mRNA was localized in the preoptic area, the anterior hypothalamus, the dorsolateral thalamus, the optic tectum and the ventral midbrain (Matsunaga et al., 2001). In addition to these diencephalic and mesencephalic regions, the expression was also localized in the septum, the hyperstriatum accessorium, the ventral portions of the archistriatum, and the cerebellar Purkinje cells (Matsunaga et al., 2001). In the cerebellum, only Purkinje cells expressed cytochrome $P450_{17\alpha,lyase}$ mRNA. Such a regional distribution of cytochrome $P450_{17\alpha,lyase}$ (Matsunaga et al., 2001) precisely correlates with that of cytochrome P450scc in the same avian species (Usui et al., 1995; Tsutsui et al., 1997a). Accordingly, both steroidogenic P450 enzymes may be co-localized in the several restricted brain regions. Indeed, cerebellar Purkinje cells possess both cytochrome P450scc (Usui et al., 1995; Tsutsui et al., 1997a,b) and cytochrome $P450_{17\alpha,lyase}$ (Matsunaga et al., 2001) in quail. A similar co-localization of cytochrome P450scc and 3β-HSD has been obtained in the rat Purkinje cell (Ukena et al., 1998, 1999b; Tsutsui and Ukena, 1999; Tsutsui et al., 1999, 2000, 2003b; Tsutsui, 2008a,b,c).

EXPRESSION, ENZYMATIC ACTIVITY, AND LOCALIZATION OF 17β-HSD IN THE AVIAN BRAIN

Androstenedione is a precursor of androgens (**Figure 6**). To determine whether androgens are synthesized in the brain independently of other steroidogenic sites, such as the gonads or adrenals, the demonstration of the presence of 17β-HSD, a key enzyme for androgen biosynthesis, is also required in the avian brain (**Figure 6**). To clarify the production of androgens in the avian brain, therefore, we examined the activity of 17β-HSD, which converts androstenedione to testosterone, using the brain of adult quail. Employing biochemical techniques combined with HPLC

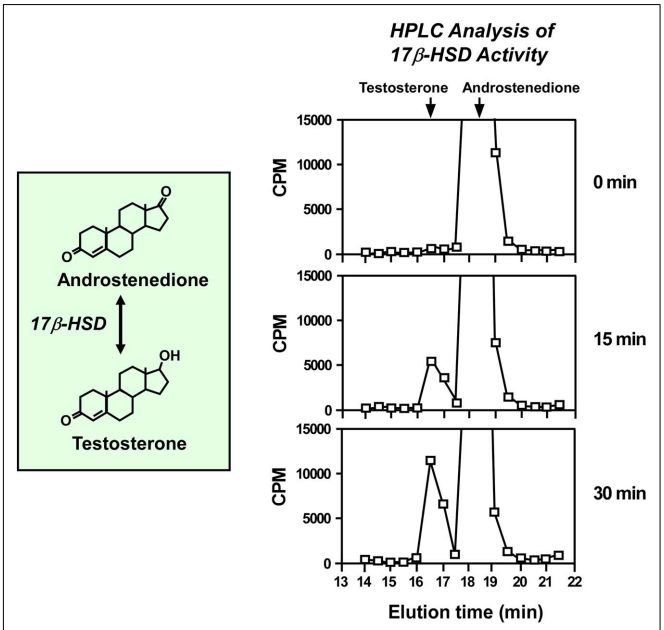

FIGURE 6 | Androgen formation from androstenedione in the quail brain. HPLC analysis of neurosteroids extracted from quail diencephalic slices after different incubation times with ^{3}H-androstenedione using a reversed-phase column. The column was eluted with a 30-min linear gradient of 40–70% acetonitrile, followed by an isocratic elution of 70% acetonitrile. The ordinate indicates the radioactivity measured in each HPLC fraction. The arrows indicate the elution positions of androstenedione and testosterone. See Matsunaga et al. (2002) for details.

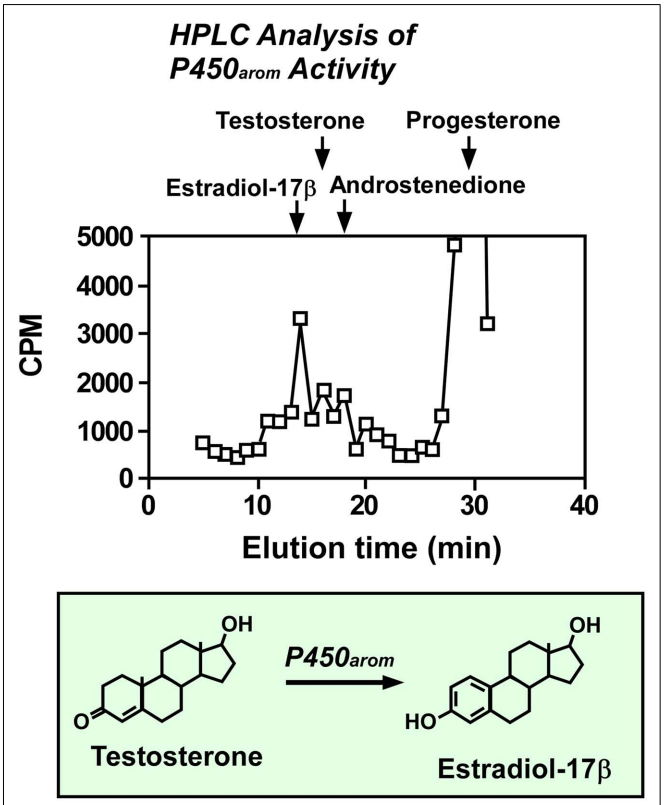

FIGURE 7 | Estrogen formation from androgen in the quail brain. HPLC analysis of neurosteroids extracted from quail diencephalic slices after 60 min incubation with ^{3}H-progesterone using a reversed-phase column. The column was eluted with a 30-min linear gradient of 40–70% acetonitrile, followed by an isocratic elution of 70% acetonitrile. The ordinate indicates the radioactivity measured in each HPLC fraction. The arrows indicate the elution positions of progesterone, androstenedione, testosterone, and estradiol-17β. See Matsunaga et al. (2002) for details.

analysis, the conversion of androstenedione to testosterone was found in quail brain slices (Matsunaga et al., 2002; **Figure 6**). Based on the biochemical analysis, we further analyzed the activity of 17β-HSD in all brain regions of the quail (Matsunaga et al., 2002). A clear difference in 17β-HSD activity among different brain regions was evident. The enzymatic activity in the diencephalon was higher than those in other brain regions (Matsunaga et al., 2002), indicating that *de novo* testosterone synthesis in the diencephalon is relatively high in birds.

EXPRESSION OF CYTOCHROME P450AROM AND ESTROGEN FORMATION IN THE AVIAN BRAIN

As mentioned above, the expression of cytochrome $P450_{17\alpha,lyase}$ and 17β-HSD and the formation of androgens have been demonstrated in the quail brain (Matsunaga et al., 2001, 2002). On the other hand, it has been well established that the brain of quail and other birds possesses cytochrome P450arom, which converts testosterone to estradiol (Schlinger and Callard, 1987, 1989a,b,c, 1991; Balthazart et al., 1990a,b, 1991; **Figure 7**). Therefore, not only androgens but also estrogens may be synthesized directly *de novo* in the avian brain. Recent progresses in the studies of physiological changes in the expression of cytochrome P450arom and their regulatory mechanisms are summarized in Section "Physiological Changes in Neurosteroid Formation and Biological Functions of Neurosteroids in the Avian Brain."

In birds, both cytochrome P450arom and estrogen receptors are expressed in several brain regions including the hypothalamus and preoptic area which are involved in the control of reproductive behaviors in birds (Schlinger and Callard, 1987, 1989a,b,c, 1991; Balthazart et al., 1990a,b, 1991). Therefore, testosterone produced in these brain regions may be converted to estradiol. In fact, we detected, biochemically, the formation of estradiol from progesterone in the quail diencephalon including the hypothalamus and preoptic area (Matsunaga et al., 2002; **Figure 7**). Taken together, testosterone is synthesized from progesterone by cytochrome $P450_{17\alpha,lyase}$ and 17β-HSD in the diencephalon, and subsequently testosterone is converted to estradiol by P450arom (**Figure 8**).

As shown in **Figure 8**, the production of sex steroids requires the coordinate action of several steroidogenic enzymes that start with cholesterol, and then lead to a cascade of reactions that ultimately produce several kinds of neurosteroids. I discuss recent studies in birds whether sex steroids are synthesized in the brain independently of other steroidogenic sites, such as the gonads and/or adrenals, or sex steroid synthesis in the brain is dependent on gonadal and/or adrenal steroids in the following Section "Physiological Changes in Neurosteroid Formation and Biological Functions of Neurosteroids in the Avian Brain."

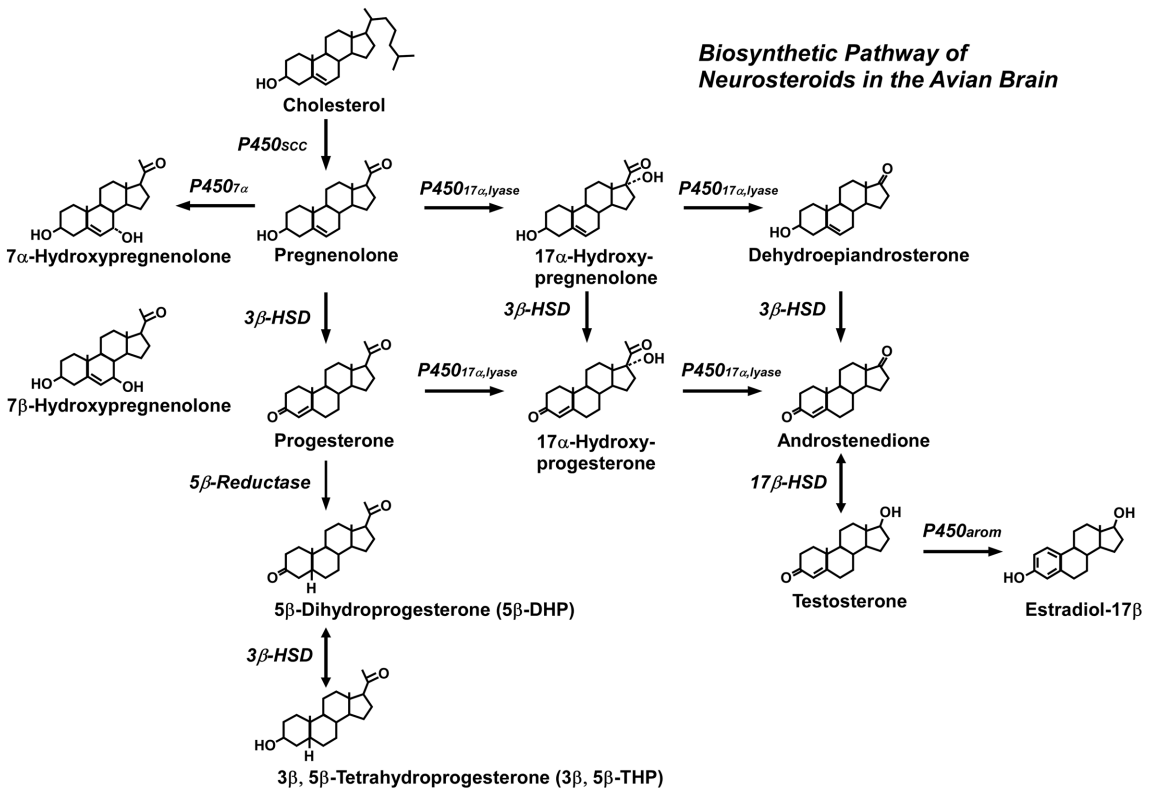

FIGURE 8 | Biosynthetic pathway for neurosteroids in the quail brain. The arrows indicate biosynthetic pathways of neurosteroids identified in the quail brain. See the text for details.

PHYSIOLOGICAL CHANGES IN NEUROSTEROID FORMATION AND BIOLOGICAL FUNCTIONS OF NEUROSTEROIDS IN THE AVIAN BRAIN

Physiological changes in neurosteroid formation and biological functions of neurosteroids are becoming clear in domestic and other birds. It is well known in birds (Tsutsui et al., 2008) as well as other vertebrates (Tsutsui, 1931; Iwata et al., 2000) that the locomotor activity of males is higher than that of females. As described in Section "Expression of Cytochrome P450$_{7\alpha}$ and Formation of 7α- and 7β-Hydroxypregnenolone in the Avian Brain," the quail brain synthesizes two previously undescribed avian neurosteroids, 7α- and 7β-hydroxypregnenolone, from pregnenolone (Tsutsui et al., 2008). In view of the sex difference in concentrations of diencephalic 7α- and 7β-hydroxypregnenolone, it seemed possible that these neurosteroids play a role in the control of locomotor activity of males. Because the male quail displays a robust locomotor activity rhythm when held under typical light/dark lighting schemes (Wilson, 1972; Wada, 1979), this bird may serve as an excellent animal model to demonstrate the biological function of 7α- and 7β-hydroxypregnenolone. Both neurosteroids were therefore administered intra-cerebroventricularly to male quail during night, when their activity is low, to test whether they affect locomotor activity (Tsutsui et al., 2008). A stimulatory dose-dependent effect of 7α-hydroxypregnenolone was observed (Tsutsui et al., 2008). In contrast, 7β-hydroxypregnenolone did not influence locomotor activity (**Figure 2**; Tsutsui et al., 2008). In contrast to males, the locomotor activity and diencephalic concentration of 7α-hydroxypregnenolone in females were constantly low during the same observation period, i.e., from lights on to noon (Tsutsui et al., 2008). Thus, increased diencephalic 7α-hydroxypregnenolone may contribute to the higher locomotor activity in males. The low level of 7α-hydroxypregnenolone synthesis and concentration in the female diencephalon suggests that this neurosteroid may not play a role in female locomotor activity. Since other vertebrates also exhibit a clear sex difference in locomotor activity (Tsutsui, 1931; Iwata et al., 2000), it is suggested that sex differences in diencephalic 7α-hydroxypregnenolone may be a key factor controlling sex differences in vertebrate locomotor activity.

A ubiquitous property of vertebrates is fluctuation of locomotor activity over the 24-h circadian cycle (Saper et al., 2005). The endogenous diurnal rhythm of melatonin is known to control the diurnal locomotor rhythm in vertebrates including 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), which suggested that melatonin may regulate diencephalic 7α-hydroxypregnenolone synthesis, and thereby influence locomotor activity. This hypothesis was tested in experiments involving melatonin manipulation in male quail (Tsutsui et al., 2008). A combination of pinealectomy (Px) plus orbital enucleation (Ex) increased the production and concentration of 7α-hydroxypregnenolone and the expression of cytochrome

$P450_{7\alpha}$ in the diencephalon. Conversely, melatonin administration to Px/Ex quail decreased the production and concentration of 7α-hydroxypregnenolone and the expression of cytochrome $P450_{7\alpha}$ in the diencephalon. Further, the inhibitory effect of melatonin on 7α-hydroxypregnenolone synthesis was abolished by luzindole, a melatonin receptor antagonist (Tsutsui et al., 2008). It is therefore considered that melatonin acts to reduce cytochrome $P450_{7\alpha}$ expression through melatonin receptor-mediated mechanisms. Melatonin derived from the pineal gland and eyes therefore appears to act as a potent inhibitory factor of 7α-hydroxypregnenolone synthesis in the quail. This hypothesis is supported by earlier studies showing that melatonin treatment decreases locomotor activity in quail (Murakami et al., 2001; Nakahara et al., 2003) and other birds (Murakami et al., 2001).

On the other hand, there is evidence for neurosteroidal activation of territorial behavior in the song sparrow *Melospiza melodia* (Soma et al., 1999, 2000). It is known that territorial behavior of this species is expressed in the non-breeding season, Although circulating testosterone levels are low in non-breeding male song sparrows, the androgen precursor dehydroepiandrosterone is present in blood (Soma and Wingfield, 2001). Because the brain of zebra finch expresses 3β-HSD and cytochrome P450arom and produces estrogens from dehydroepiandrosterone (Vanson et al., 1996; Soma et al., 2004; Tam and Schlinger, 2007), it is considered that these steroidogenic enzymes may function in the brain of song sparrow during the non-breeding season to produce estrogens from dehydroepiandrosterone originated from the peripheral gland that activate territorial aggression. Because the brain of zebra finch also expresses cytochrome P450scc and cytochrome $P450_{17\alpha,lyase}$ (London et al., 2003, 2006, 2010; London and Schlinger, 2007), dehydroepiandrosterone may also be produced *de novo* from cholesterol in the brain of these birds as in quail. More research is needed to evaluate the function of neurosteroids produced in the brain from cholesterol *de novo* and the role of central metabolism of steroids originally coming from the periphery.

As was detected in zebra finches, song sparrows expressed 3β-HSD and cytochrome P450arom in the brain (Soma et al., 2003; Pradhan et al., 2008, 2010). Importantly, both steroidogenic enzymes are subject to seasonal regulation. Cytochrome P450arom is elevated in the non-breeding season in the brain in keeping with a role for estrogen activation of aggressive behavior during the non-breeding season (Soma et al., 2003). 3β-HSD is expressed and active in the song sparrow brain (Pradhan et al., 2008, 2010). 3β-HSD is also elevated during the non-breeding season. It is important to clarify the mechanisms that regulate the expression of these steroidogenic enzymes in the avian brain.

Interestingly, there are several reports showing changes in neurosteroid formation in relation to social interactions. A recent study showed that within the caudomedial nidopallium marked changes in estradiol occurred when males were exposed to females or to conspecific zebra finch song (Remage-Healey et al., 2008). Estrogens produced in the local brain region are thought to rapidly strengthen auditory encoding and guide song preference in a songbird (Remage-Healey et al., 2010). Moreover, changes in estradiol formation were reduced by exposure to fadrozole, an inhibitor of cytochrome P450arom, or to glutamate as in quail hypothalamus (Balthazart et al., 2006). These findings suggest rapid control of cytochrome P450arom activity by glutamatergic inputs (Balthazart et al., 2006). Thus, cytochrome P450arom is subject to rapid regulation. In quail hypothalamic explants, cytochrome P450arom undergoes Ca^{2+}-dependent phosphorylation that reduces cytochrome P450arom activity within minutes (Balthazart et al., 2001a,b, 2003). Treatments of these explants with K^+ or with glutamate receptor agonists produce a similar rapid inhibition of cytochrome P450arom activity (Balthazart et al., 2001b). These results suggest that the flow of Ca^{2+} through voltage-gated Ca^{2+} channels serves as a key regulatory signal for rapid estrogen production.

Synaptic estrogen formation in the brain is also becoming clear in songbirds and other birds (Naftolin et al., 1996; Peterson et al., 2005) as in mammals (Hojo et al., 2008). Compartmentalization of cytochrome P450arom within presynaptic boutons is considered to be crucial for providing sex- and song-specific estrogenic signals in the songbird brain (Peterson et al., 2005; Remage-Healey et al., 2009).

MODE OF ACTION OF NEUROSTEROIDS IN THE AVIAN BRAIN

To understand the mode of action of 7α-hydroxypregnenolone on locomotion, Matsunaga et al. (2004) first found that 7α-Hydroxypregnenolone acts as a neuronal activator to stimulate locomotor activity of breeding newts by means of the dopaminergic system (Matsunaga et al., 2004). As described in Section "Expression of Cytochrome $P450_{7\alpha}$ and Formation of 7α- and 7β-Hydroxypregnenolone in the Avian Brain," the expression of cytochrome $P450_{7\alpha}$ mRNA was localized in several diencephalic regions, such as the nucleus preopticus medialis, the nucleus paraventricularis magnocellularis, the nucleus ventromedialis hypothalami, the nucleus dorsolateralis anterior thalami, and the nucleus lateralis anterior thalami (Tsutsui et al., 2008) in the male quail brain. In quail (Tsutsui et al., 2008) as in newts (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 area ventralis and the substantia nigra, project to the telencephalon notably the striatum (Mezey and Csillag, 2002; Hara et al., 2007). Interestingly, the telencephalic region is enriched with dopamine D_1 and D_2 receptors in birds (Ball et al., 1995; Levens et al., 2000). Accordingly, 7α-hydroxypregnenolone synthesized actively in the diencephalon, by acting on dopamine neurons localized in the area ventralis and the substantia nigra, may induce dopamine release from their termini in the striatum, and consequently increase locomotor activity in male quail as in male newts.

The fact that 7α-hydroxypregnenolone acutely increases locomotor activity in quail suggests that this neurosteroid may act through a non-genomic rather than a genomic mechanism. It has been reported that in rats, the progesterone metabolite 3α,5α-tetrahydroprogesterone (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 quail are mediated through $GABA_A$ receptors remain to be determined.

On the other hand, there are several reports showing the mode of acute actions of estrogen (Tremere et al., 2009; Remage-Healey et al., 2010; Tremere and Pinaud, 2011). The neuromodulatory role of estradiol on burst firing of neurons in the caudomedial nidopallium was demonstrated in the songbird (Tremere and Pinaud, 2011). Acute estrogen actions in the zebra finch may be dependent on a membrane-specific receptor. Further study is needed to demonstrate the molecular mechanisms underlying acute actions of estrogen in the brain of birds.

CONCLUSION AND FUTURE DIRECTIONS

In conclusion, the quail brain possesses several kinds of steroidogenic enzymes, such as cytochrome P450scc, cytochrome $P450_{7\alpha}$, 3β-HSD, 5β-reductase, cytochrome $P450_{17\alpha,lyase}$, 17β-HSD and cytochrome P450arom, and produces pregnenolone, 7α-hydroxypregnenolone, progesterone, 5β-DHP, 3β, 5β-THP, androstenedione, testosterone, and estradiol from cholesterol (**Figure 8**). The brain of other birds, such as ring dove, zebra finch etc., also produces several neurosteroids. However, the biosynthetic pathway of neurosteroids in the avian brain from cholesterol may be still incomplete, because we recently found that the quail brain expresses cytochrome $P450_{7\alpha}$ and actively produces 7α- and 7β-hydroxypregnenolone, previously undescribed avian neurosteroids (**Figure 8**). Thus, to complete our knowledge of the biosynthetic pathway for neurosteroids in the avian brain, further biochemical studies are needed.

Diurnal and seasonal changes in neurosteroid formation are becoming clear in birds. Social interactions also change neurosteroid formation in birds. Further, several important biological functions of neurosteroids are also becoming clear in birds. However, more research is needed to evaluate the function of neurosteroids produced in the brain from cholesterol *de novo* and the role of central metabolism of steroids originally coming from the periphery. In addition, it is important to clarify the molecular mechanisms of the regulation of neurosteroid formation and the mode of action of neurosteroids in the avian brain.

ACKNOWLEDGMENTS

The author thanks Mariko Usui, Takeshi Yamazaki, Shiro Kominami, Kazuyoshi Ukena, Yoko Honda, Masahiro Matsunaga, Hirotaka Sakamoto, Yuto Inai, Minoru Takase, Chie Kohchi, Kazuhiko Inoue, Hitomi Miyabara, Takayoshi Ubuka, and Shogo Haraguchi for their work cited in this manuscript. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (18107002, 22132004, and 22227002 to Kazuyoshi Tsutsui).

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Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 May 2011; paper pending published: 13 June 2011; accepted: 05 September 2011; published online: 21 September 2011.

*Citation: Tsutsui K (2011) Neurosteroid biosynthesis and function in the brain of domestic birds. Front. Endocrin. **2**:37. doi: 10.3389/fendo.2011.00037*

This article was submitted to Frontiers in Neuroendocrine Science, a specialty of Frontiers in Endocrinology.