Neurosteroid biosynthesis in the brain of amphibians

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INTRODUCTION
Steroid hormones play a crucial role in the development, growth, and differentiation of the central nervous system (CNS; McEwen, 1992; Compagnone and Mellon, 2000; Sakamoto et al., 2001; Karishma and Herbert, 2002; Wang et al., 2005; Lazaridis et al., 2011). Because of their lipophilic nature, circulating steroids can readily cross the blood–brain barrier, and it has long been assumed that the adrenal gland, gonads, and placenta were the sole sources of steroid hormones acting on the brain. However, in the early 1980s, Baulieu, Robel, and co-workers made a series of seminal observations which revealed that this view was not correct. They initially found that the concentrations of several steroids, including pregnenolone (Δ5P), dehydroepiandrosterone (DHEA), and their sulfate esters, were much higher in the central and peripheral nervous systems than in the plasma (Corpéchot et al., 1981, 1983). They then reported that the level of certain brain steroids remained elevated after adrenalectomy and castration, and that ACTH treatment, although increasing plasma Δ5P concentration in brain tissue (Corpéchot et al., 1981, 1983; Robel and Baulieu, 1985; Cheney et al., 1995). These observations led them to hypothesize that the brain had the capability of synthesizing de novo biologically active steroids. In support of this hypothesis, the presence of steroidogenic enzymes has now been visualized by immunohistochemistry and in situ hybridization, either in neurons or in glial cells, in the brain of various representative vertebrate species, and the occurrence of the corresponding enzymatic activities has been ascertained through biochemical approaches (Mensah-Nyagan et al., 1999; Tsutsui et al., 1999; Compagnone and Mellon, 2000; Mellon and Vaudry, 2001; Do Rego et al., 2009).

Concurrently, it has been shown that brain-born steroids (now called neurosteroids) exert pleiotropic activities in the CNS. Neurosteroids act either via conventional nuclear receptors or via allosteric modulation of membrane receptors, e.g., GABA_A, glycine, NMDA, kainate, AMPA, sigma, nicotinic, muscarinic, and serotoninergic receptors (Majewska, 1992; Belelli and Lambert, 2005; Belelli et al., 2006; Herd et al., 2007).

The amphibian brain is a very convenient model to study neurosteroidogenesis. Firstly, most enzymes of the steroidogenic pathways are expressed in the CNS of amphibians. Secondly, the activity of the steroidogenic enzymes in the brain of amphibians is very high, thus making it possible to investigate the effects of inhibitory factors regulating neurosteroidogenesis. Thirdly, because of its relatively small size, the brain of amphibians is particularly suitable for studying the neuroanatomical distribution of neurosteroid-producing cells. The present report summarizes the current knowledge concerning the occurrence, distribution, and biological activity of steroidogenic enzymes in the brain of amphibians. A companion paper describes the neuronal mechanisms involved in the regulation of neurosteroidogenesis in amphibians (Do Rego et al., submitted).
**TRANSLOCATOR PROTEIN**

Translocator protein (TSPO) is an 18-kDa protein that is part of the peripheral benzodiazepine receptor complex (Papadopoulos et al., 2006). TSPO, which is primarily located on the outer mitochondrial membrane, binds with high affinity cholesterol and enhances its translocation to the inner mitochondrial membrane (Papadopoulos et al., 1997; Rupprecht et al., 2009). In the brain of the European green frog *Rana esculenta*, the distribution of TSPO has been determined by immunohistochemistry using polyclonal antibodies against a synthetic fragment of the human protein (Oke et al., 1992). TSPO-immunoreactive cells are located in several nuclei of the telencephalon and diencephalon including the anterior commissure, the lateral and medial pallium, the lateral septum, the medial amygdala, the anterior preoptic area, the dorsal hypothalamic nucleus, the posterior tuberculum, the suprachiasmatic nucleus, and the ventral habenular nucleus (Do Rego et al., 1998). Interestingly, in several of these nuclei, TSPO is co-localized with the steroidogenic enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) supporting the involvement of TSPO in the biosynthesis of neurosteroids in the frog brain (Do Rego et al., 1998).

**STEROID ACUTE REGULATORY PROTEIN**

Steroid acute regulatory protein (StAR) is a 30-kDa protein that plays a major role in the transfer of cholesterol from the outer to the inner membrane of mitochondria (Stocco, 2001). StAR mRNA is actively expressed in the brain of mammals including human (Furukawa et al., 1998; Inoue et al., 2002). In the human frontal cortex, immunohistochemical studies have shown that StAR and cytochrome P450 side-chain cleavage (P450scc) co-exist in a discrete population of neurons. The expression of the StAR gene has also been evidenced in the brain of birds (London et al., 2006) and fish (Nunez et al., 2005; Lyssimachou and Arukwe, 2007; Arukwe, 2008; Kusakabe et al., 2009). However, up to now, the occurrence of StAR has not yet been investigated in the frog brain (Do Rego et al., 2009).

**CYTOCHROME P450 SIDE-CHAIN CLEAVAGE**

Cytochrome P450scc (CYP11A1), a member of the cytochrome P450 superfamily of enzymes, is located on the inner mitochondrial membrane (Farkash et al., 1986). P450scc is a rate-limiting and hormonally regulated enzyme that catalyzes the conversion of cholesterol into Δ5P, the universal precursor of all steroid hormones (Guo et al., 2007; Lavoie and King, 2009; Figure 1). P450scc was the first steroidogenic enzyme whose presence was detected in the CNS (Le Goascogne et al., 1987). Since then, the occurrence of P450scc has been reported in the brain of rodents (Compagnone et al., 1995a; Ukena et al., 1998; Kimoto et al., 2001) and primates (Le Goascogne et al., 1989) as well as in birds (Tsutsui and Yamazaki, 1995; Usui et al., 1995; Lea et al., 2001; London et al., 2006). In amphibians, the localization of P450scc has been described in both anurans and urodeles. In the clawed toad *Xenopus laevis* and the frog *Rana nigromaculata*, P450scc is particularly

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**FIGURE 1 | Biochemical pathways for the synthesis of neurosteroids.** The color code is the same as that used in Figures 2–6. Abbreviations for steroids: B, corticosterone; C, cholesterol; CS, cholesterol sulfate; Δ4, androstenedione; Δ4P, pregnenolone; Δ4PS, pregnenolone sulfate; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHDOC, dihydrolutein; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DHHDOC, dihydrolutein; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; THDOC, tetrahydrodeoxycorticosterone; THP, tetrahydroprogesterone; 3α-diol, androstenediol; 7α-OH-Δ4P; 7α-hydroxyprogrenolone; 7α-OH-DHEA, 7α-hydroxydehydroepiandrosterone; 11-DOC, 11-deoxycorticosterone; 17OH-Δ2P, 17-hydroxyprogrenolone; 17OH-Δ2P, 17-hydroxyprogesterone. Abbreviations for enzymes: AKR1C, 3α-hydroxysteroid dehydrogenase; CYP7A1, oxysterol 7α-hydroxylase (cytochrome P450scc); CYP11A1, cytochrome P450 side-chain cleavage; CYP11B1, 11β-hydroxylase (cytochrome P450scc); CYP17A1, cytochrome P450 P450 17α-hydroxylase/C17, 20-lyase; CYP19A1, cytochrome P450 aromatase; CYP21, 21-hydroxylase (P450c21); HSD25B, 3β-hydroxysteroid dehydrogenase/Δ5-Δ4 isomerase; HSD17B, 17β-hydroxysteroid dehydrogenase; SRD5A, 5α-reductase; STS, sulfatase; SULT2, hydroxysteroid sulfotransferase.
abundant in the ventral hypothalamic nucleus and the medial pallium (Takase et al., 1999). P450sc-immunoreactivity is also present in the anterior preoptic area, the dorsal and lateral pallium, the dorsal striatum, the ventral part of the magnocellular preoptic nucleus, the optic tectum, the pretectal gray, Purkinje cells in the cerebellum, and lamina six (Takase et al., 1999). In R. esculenta, P450sc-immunoreactivity is found in both neurons and glial cells (unpublished observations). The fact that the concentrations of Δ5P in the brain of X. laevis (Takase et al., 1999), R. esculenta (Mensah-Nyagan et al., 1994), and the red-bellied newt Cynops pyrrhogaster (Inai et al., 2003) are higher than in the plasma and even the gonads suggests that the P450sc-immunoreactive protein detected in the brain of these amphibian species actually corresponds to a biologically active form of the enzyme. In support of this hypothesis, a recent study has shown the conversion of cholesterol into Δ3P in the newt brain (Takase et al., 2011).

3β-HYDROXYSTEROID DEHYDROGENASE

3β-Hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase (3β-HSD; HSD3B) is a membrane bound enzyme that catalyzes the conversion of Δ3β-hydroxysteroids into Δ4-3-ketosteroids (Figure 1). This enzyme is thus essential for the biosynthesis of all classes of steroid hormones including progesterone (P), glucocorticoids, mineralocorticoids, androgens, and estrogens (Payne and Hales, 2004; Simard et al., 2005). The existence of 3β-HSD mRNA and/or protein has been documented in the brain of mammals (Dupont et al., 1994; Guennoun et al., 1995; Sanne and Krueger, 1995; Ukena et al., 1999a) and birds (Ukena et al., 1999b, 2001). In amphibians, the neuroanatomical distribution of 3β-HSD immunoreactivity has been described in the CNS of R. esculenta (Mensah-Nyagan et al., 1994) and C. pyrrhogaster (Inai et al., 2003). In adult frog, 3β-HSD-containing neurons are exclusively located in the diencephalon, namely in the anterior preoptic area and the dorsal and ventral hypotalamic nuclei (Figure 2). These neurons send projections in the telencephalon, notably in the nucleus accumbens, and in various nuclei of the diencephalon (Mensah-Nyagan et al., 1994). During early development (stages IV–XII), intense 3β-HSD immunoreactivity is found in the dorsal part of the anterior olfactory nucleus (Bruzone et al., 2010). The concentration of P in the frog hypothalamus is about 200-fold higher than in the plasma (Mensah-Nyagan et al., 1994). Likewise, in the brain of the toad X. laevis and the newt C. pyrrhogaster, P levels are higher than in the plasma and gonads (Takase et al., 1999; Inai et al., 2003) suggesting that brain P does not originate from steroidogenic glands. Consistent with this notion, incubation of frog hypothalamic explants or newt brain homogenates with tritiated pregnenolone ([3H]P, mensah-Nyagan et al., 1994; Inai et al., 2003). Taken together, these observations indicate that 3β-HSD immunoreactivity detected in the brain of amphibians is biologically active.

CYTOCHROME P4507A

Cytochrome P4507A (P4507A; CYP7A1) is a microsomal enzyme with broad substrate specificity for sterol metabolites (Gilardi et al., 2007; Norlin and Wikvall, 2007; Figure 1). One of the isoforms of P4507A, CYP7B, is expressed in the brain of mammals (Stapleton et al., 1995) and birds (Tatsusi et al., 2008). The first evidence for the presence of P4507A in the brain of amphibians was provided by Matsunaga et al. (2004) who showed that the brain of the newt C. pyrrhogaster actively converts [3H]Δ3P into tritiated 7α-hydroxypregnenolone ([3H]7α-OH-Δ3P). Subsequently, CYP7B was cloned from the newt brain confirming the occurrence of P4507A in the CNS of amphibians (Haraguchi et al., 2010). Newt CYP7B-transfected COS-7 cells can catalyze the conversion of [3H]Δ3P into [3H]7α-OH-Δ3P, and the enzymatic activity is blocked by the cytochrome P450 inhibitor ketoconazole (Haraguchi et al., 2010). In the newt brain, CYP7B-immunoreactive cells are primarily located in the anterior preoptic area, the magnocellular preoptic nucleus, and the tegmental area. Immunoreactive cells are also found in the lateral and dorsal pallium, the suprachiasmatic nucleus, the ventral hypothalamic nucleus, and the tectum mesencephali (Haraguchi et al., 2010). Recent studies have shown that the synthesis of 7α-OH-Δ3P in the newt brain undergoes marked diurnal and seasonal variations in parallel with changes in locomotor activity (Matsunaga et al., 2004; Tsutsui et al., 2008, 2010a,b; Haraguchi et al., 2009, 2010). The observation that 7α-OH-Δ3P biosynthesis in the newt brain is stimulated by melatonin (Tsutsui et al., 2010a) and prolactin (Haraguchi et al., 2010) strongly suggests that these two factors are responsible, respectively, for the diurnal and seasonal variations in 7α-OH-Δ3P formation. The functional significance of 7α-OH-Δ3P in the control of locomotor activity is discussed in a companion paper (Haraguchi et al., 2011).

CYTOCHROME P450 17α-HYDROXYLASE/C17,20-LYASE

The microsomal enzyme cytochrome P450 17α-hydroxylase/C17, 20-lyase (P450c17, CYP17A1) possesses 17α-hydroxylase activity and can thus convert Δ5P into 17-hydroxypregnenolone (17OH-Δ5P) and P into 17-hydroxyprogesterone (17OH-P; Figure 1). P450c17 also possesses 17,20-lyase (P450C17; Haraguchi et al., 2010) and prolactin (Haraguchi et al., 2010) strongly suggests that these two factors are responsible, respectively, for the diurnal and seasonal variations in Δ3P formation. The functional significance of Δ3P in the control of locomotor activity is discussed in a companion paper (Haraguchi et al., 2011).
(Le Gascogne et al., 1991; Mellon and Deschepper, 1993; Compagnone et al., 1995b; Yamada et al., 1997; Hojo et al., 2004). In contrast, the occurrence of P450c17 has been unequivocally demonstrated in the CNS of birds (Matsunaga et al., 2001) and amphibians (Do Rego et al., 2007). In R. esculenta, P450c17 immunoreactivity is essentially located in neurons, notably in the diencephalon, but a few P450c17-positive glial cells are also observed in the mesencephalon (Do Rego et al., 2007). Thus, a dense accumulation of P450c17-immunoreactive neurons is found in the anterior preoptic area, the ventral hypothalamic nuclei, the magnocellular nuclei, the posterior tuberculum, the habenular nuclei, and the nucleus of the periventricular organ. P450c17-positive neurons are also present in the olfactory bulb, the lateral amygdala and the medial pallium (Do Rego et al., 2007; Figure 3). Frog hypothalamic explants are capable of converting [3H]Δ3P into C21 17-hydroxysteroids and C19 ketosteroids such as 17-OH-DHT (Bruzzone et al., 2010). In the diencephalon, but a few P450C17-positive glial cells are also observed in the mesencephalon (Do Rego et al., 2007). Thus, a dense accumulation of P450c17-immunoreactive neurons is found in the anterior preoptic area, the ventral hypothalamic nuclei, the magnocellular nuclei, the posterior tuberculum, the habenular nuclei, and the nucleus of the periventricular organ. P450c17-positive neurons are also present in the olfactory bulb, the lateral amygdala and the medial pallium (Do Rego et al., 2007; Figure 3). Frog hypothalamic explants are capable of converting [3H]Δ3P into C21 17-hydroxysteroids and C19 ketosteroids such as 17-OH-DHT (Bruzzone et al., 2010; Figure 3). 3α-Hydroxysteroid oxidoreductase, also called 3α-hydroxysteroid dehydrogenase (3α-HSD; AKR1C) is a microsomal enzyme that catalyzes in a reversible manner the conversion of 3α-DHP and 5α-DHT into THP (allopregnanolone) and 3α-androstenediol, respectively (Figure 1). 3α-HSD is also involved in the reversible conversion of dihydrodeoxycorticosterone (DHDOC) into tetrahydrodeoxycorticosterone (THDOC; Penning et al., 2006; Figure 1). A single form of 3α-HSD exists in rodents (Pawlowski et al., 1991) while four isoforms have been characterized in humans (Khanna et al., 1995). The distribution of 3α-HSD mRNA and protein has been described in the brain of human (Griffin and Mellon, 1999; Steckelbroeck et al., 2001) and rodents (Khanna et al., 1995; Agis-Balboa et al., 2006), but has not yet been reported in amphibians. Using an antibody against human 3α-HSD, we have recently observed the existence of 3α-HSD-immunoreactive neurons in the brain of R. esculenta. Positive cells are found in the olfactory bulb, the anterior commissure, the anterior preoptic area, the magnocellular preoptic nucleus, the ventral hypothalamic nuclei, the posterior tuberculum (unpublished observations). We have also seen that frog hypothalamic explants can synthesize THP from [3H]Δ3P (unpublished observations), indicating that a bioactive form of 3α-HSD is actually present in the brain of amphibians. 17β-Hydroxysteroid dehydrogenase 17β-Hydroxysteroid dehydrogenase (17β-HSD; HSD17B) is a bifunctional enzyme that catalyzes the interconversion of 17-ketosteroids and 17β-hydroxysteroids (Payne and Hales, 2004). 17β-HSD thus plays a pivotal role in the biosynthesis and inactivation of sex steroid hormones (Figure 1). Twelve isoforms of 17β-HSD exhibiting different substrate specificity, tissue expression, and subcellular localization have been characterized in vertebrates (Luu-The, 2001; Luu-The et al., 2006). The occurrence of 17β-HSD
has been demonstrated in the brain of mammals (Pelletier et al., 1995; Hojo et al., 2004) and birds (Nomura et al., 1998; Matsumaga et al., 2002). In amphibians, immunohistochemical localization of 17β-HSD has been determined in the CNS of the frog Rana ridibunda using polyclonal antibodies against type I human placental 17β-HSD (Dupont et al., 1991). In this species, 17β-HSD immunoreactivity is found in a discrete population of glial cells. Positive glioocytes are located in the telencephalon, i.e., the periventricular zone of the medial pallium, and in the rostral region of the diencephalon (Mensah-Nyagan et al., 1996a,b; Figure 5). The presence of substantial amounts of T in the brain of both male and female frogs has been shown by gas chromatography–mass spectrometry and by HPLC analysis combined with radioimmunoassay (Mensah-Nyagan et al., 1996a,b). In male frogs, castration does not affect the concentration of T and 5α-DHT in the telencephalon and hypothalamus (Mensah-Nyagan et al., 1996b). Finally, in vitro studies have demonstrated that frog telencephalon explants can convert [3H]Δ5P into [3H]T and [3H]5α-DHT (Mensah-Nyagan et al., 1996a,b). Altogether, these data provide strong evidence for the existence of a bioactive form of 17β-HSD in the forebrain of amphibians and for the biosynthesis of sex steroids from Δ5P by glial cells of the telencephalon and diencephalon.

CYTOCHROME P450AROMATASE

Cytochrome P450 aromatase (P450arom; CYP19A1) is a microsomal enzyme that catalyzes the transformation of androgens into estrogens (Simpson et al., 2002; Figure 1). It has long been established that aromatization of androgens is required for their regulatory effects in the CNS (MacLusky and Naftolin, 1981). Thus, P450arom plays a crucial role in the sexual differentiation of the brain and in the regulation of reproductive functions (Garcia-Segura, 2008). The distribution of P450arom has been extensively investigated in the brain of mammals (Roselli et al., 1985; Baltz hazart et al., 1991; Abdelgadir et al., 1997) and birds (Balthazart et al., 1990; Ervard et al., 2004). The occurrence and localization of P450arom has also been described in the brain of teleost fish (Callard et al., 1978; Gelinhas and Callard, 1997; Goto-Kazeto et al., 2004; Diotel et al., 2010). In amphibians, the presence of P450arom has been reported in the brain of Necturus maculosus (Callard et al., 1978) and R. esculenta (Guerrero et al., 2000). The expression of P450arom mRNA in the brain of Pleurodiles waltl (Kuntz et al., 2004) and X. laevis (Urbatza et al., 2007) has been investigated during development. In both species, the P450arom gene is expressed from early developmental stages to metamorphosis without noticeable differences between males and females. However, the precise neuroanatomical distribution of P450arom mRNA or protein has not yet been determined in any amphibian species. Incubation of frog hypothalamic homogenates with [3H]DHEA induces the formation of estrone sulfate (Beaujean et al., 1999). Similarly, incubation of frog hypothalamic explants with [3H]Δ5P yields to the biosynthesis of estradiol (Mensah-Nyagan et al., 1996a,b). Taken together, these observations indicate that, in amphibians as in other vertebrate groups, an active form of aromatase is present in the brain.

HYDROXYSTEROID SULFOTRANSFERASE

Hydroxysteroid sulfotransferase (HST; SULT2) designates a family of cytosolic enzymes that transfer the sulfonate moiety from 3′-phosphoadenosinophosphate 5′-phosphosulfate (PAPS) on the 3-hydroxyl or the 21-hydroxyl group of steroid substrates (Strott, 2002; Figure 1). The observation that 3-hydroxysteroid sulfonates, such as pregnenolone sulfate (Δ5PS) and dehydroepiandrosterone sulfate (DHEAS), act as allosteric modulators of various receptors for neurotransmitters, notably GABAa receptors (Majewska, 1992; Le Foll et al., 1997) indicates that HST plays a critical role in the bioactivation of certain neurosteroids. Remarkably, while in mammals, immunohistochemical studies failed to detect HST in the brain (Sharp et al., 1993), in amphibians, the occurrence of HST immunoreactivity has been thoroughly investigated in R. ridibunda (Beaujean et al., 1999). Using an antiserum against rat liver HST, two populations of HST-immunoreactive neurons have been visualized in the dorsal part of the magnocellular preoptic nucleus and the anterior preoptic area (Figure 6). These neurons send projections in various regions of the telencephalon, diencephalon, and mesencephalon, notably in the medial amygdala, medial pallium, medial septum, nucleus accumbens, nucleus of the diagonal band of Broca, anterior thalamic nucleus, posterior thalamic nucleus, anterior preoptic area, basal optic nucleus, and nucleus reticularis isthmi (Beaujean et al., 1999). Incubation of frog telencephalon or hypothalamus homogenates with [3H]Δ5P or [3H]DHEA as steroid precursors and [35S]PAPS as a sulfate donor yields the formation of several 3H, 35S-labeled steroids including Δ5PS, DHEAS, and testosterone sulfate (TS; Beaujean et al., 1999). De novo synthesis of 3-hydroxysteroid sulfate is blocked by the sulfotransferase inhibitor 2,4-dichloro-6-nitrophenol (Beaujean et al., 1999). These data demonstrate that the brain of amphibians
expresses HST and has the ability to synthesize the highly potent neurosteroids Δ5PS and DHEAS.

CONCLUDING REMARKS

Amphibians have been widely used as animal models to investigate neurosteroid biosynthesis in the brain. The neuroanatomical distribution of most key steroidogenic enzymes has now been determined in the brain of anurans and/or urodeles. For some of these enzymes, e.g., 3β-HSD and HST, the immunohistochemical localization in the CNS has been first reported in amphibians. Biochemical studies have demonstrated that these enzymes are able to convert radiolabeled steroid precursors such as [3H]Δ5P or [3H]DHEA into biologically active steroids. The occurrence of high concentrations of steroids in brain tissue confirms that amphibians have the ability to synthesize neurosteroids which are, at least in part, produced independently of steroidogenic glands. The fact that amphibians and mammals, which diverged approximately 350 million years ago, have in common the ability to synthesize de novo biologically active steroids in their brain supports the view that neurosteroids are playing vital functions throughout the animal kingdom.

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