



Regulation of neurosteroid biosynthesis by neurotransmitters and neuropeptides

Jean Luc Do Rego^{1,2,3}, Jae Young Seong⁴, Delphine Burel^{1,2,3,5}, Jérôme Leprince^{1,2,3,5}, David Vaudry^{1,2,3,5}, Van Luu-The⁶, Marie-Christine Tonon^{1,2,3,5}, Kazuyoshi Tsutsui^{7,8}, Georges Pelletier⁶ and Hubert Vaudry^{1,2,3,5*}

¹ INSERM, Mont-Saint-Aignan, France

² European Institute for Peptide Research, IFRMP 23, Regional Platform for Cell Imaging, PRIMACEN, University of Rouen, Mont-Saint-Aignan, France

³ International Associated Laboratory Samuel de Champlain, Mont-Saint-Aignan, France

⁴ Laboratory of G Protein-Coupled Receptors, Graduate School of Medicine, Korea University College of Medicine, Seoul, Korea

⁵ Laboratory of Neuronal and Neuroendocrine Differentiation and Communication, INSERM U982, University of Rouen, Mont-Saint-Aignan, France

⁶ Research Center in Molecular Endocrinology, Oncology and Genetics, Laval University Hospital Center, Québec, QC, Canada

⁷ Laboratory of Integrative Brain Sciences, Department of Biology, Waseda University, Tokyo, Japan

⁸ Center for Medical Life Science of Waseda University, Tokyo, Japan

Edited by:

Olivier Kah, CNRS UMR 6026, France

Reviewed by:

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James A. Carr, Texas Tech University,

USA

*Correspondence:

Hubert Vaudry, INSERM U982,

European Institute for Peptide

Research, IFRMP 23, University of

Rouen, 76821 Mont-Saint-Aignan,

France.

e-mail: hubert.vaudry@univ-rouen.fr

The enzymatic pathways leading to the synthesis of bioactive steroids in the brain are now almost completely elucidated in various groups of vertebrates and, during the last decade, the neuronal mechanisms involved in the regulation of neurosteroid production have received increasing attention. This report reviews the current knowledge concerning the effects of neurotransmitters, peptide hormones, and neuropeptides on the biosynthesis of neurosteroids. Anatomical studies have been carried out to visualize the neurotransmitter- or neuropeptide-containing fibers contacting steroid-synthesizing neurons as well as the neurotransmitter, peptide hormones, or neuropeptide receptors expressed in these neurons. Biochemical experiments have been conducted to investigate the effects of neurotransmitters, peptide hormones, or neuropeptides on neurosteroid biosynthesis, and to characterize the type of receptors involved. Thus, it has been found that glutamate, acting through kainate and/or AMPA receptors, rapidly inactivates P450arom, and that melatonin produced by the pineal gland and eye inhibits the biosynthesis of 7 α -hydroxypregnenolone (7 α -OH- Δ^5 P), while prolactin produced by the adenohypophysis enhances the formation of 7 α -OH- Δ^5 P. It has also been demonstrated that the biosynthesis of neurosteroids is inhibited by GABA, acting through GABA_A receptors, and neuropeptide Y, acting through Y1 receptors. In contrast, it has been shown that the octadecaneuropeptide ODN, acting through central-type benzodiazepine receptors, the triakontatetrapeptide TTN, acting through peripheral-type benzodiazepine receptors, and vasotocin, acting through V1a-like receptors, stimulate the production of neurosteroids. Since neurosteroids are implicated in the control of various neurophysiological and behavioral processes, these data suggest that some of the neurophysiological effects exerted by neurotransmitters and neuropeptides may be mediated via the regulation of neurosteroid production.

Keywords: neurosteroids, glutamate, GABA, melatonin, prolactin, endozepines, vasopressin, neuropeptide Y

INTRODUCTION

The regulatory effects of steroid hormones in the central nervous system (CNS) have long been recognized (McEwen, 1994; Baulieu et al., 1999; Dubrovsky, 2005; Leskiewicz et al., 2006). Steroids acting on the CNS originate from two sources: (1) steroids produced by peripheral endocrine glands, i.e., gonads, adrenal, and placenta, that have to cross the blood–brain barrier to act on the brain, are usually designated as *neuroactive steroids*; (2) steroids directly synthesized within the CNS, either *de novo* from cholesterol or by *in situ* metabolism of circulating steroid precursors, are designated by the generic term *neurosteroids* (Robel and Baulieu, 1985, 1994; Baulieu, 1997, 1998). The capability of the nervous system to synthesize steroids was originally discovered in mammals (Corpéchet et al., 1981, 1983; Lanthier and Patwardhan, 1986)

and was subsequently generalized in other vertebrates including birds, amphibians, and fish (Mensah-Nyagan et al., 1999; Mellon and Vaudry, 2001; Tsutsui et al., 2003, 2009; Do Rego et al., 2009; Diotel et al., 2010) indicating that *de novo* neurosteroidogenesis is a conserved property in the vertebrate phylum.

There is growing evidence that neurosteroids play an important role as endogenous modulators of neuronal functions and behavioral processes, and that alterations of neurosteroid concentrations may contribute to the pathophysiology of neuronal disorders (Majewska, 1992; Robel et al., 1999; Rupprecht and Holsboer, 1999; Lapchak et al., 2000; Lapchak and Araujo, 2001; Rupprecht et al., 2001; Dubrovsky, 2005; Belelli et al., 2006; Strous et al., 2006). For instance, in rat, infusion of pregnenolone sulfate (Δ^5 PS) and dehydroepiandrosterone sulfate (DHEAS) into

the nucleus basalis magnocellularis enhances learning and memory (Mayo et al., 1993; Robel et al., 1995). Reciprocally, deficit in cognitive performances in aged rats and mice is correlated with low Δ^5 PS and DHEAS levels in the hippocampus (Flood et al., 1988, 1992; Vallée et al., 1997, 2001; Ladurelle et al., 2000). In chickens, administration of DHEA and DHEAS enhances learning and memory (Migues et al., 2002). In humans, DHEA and DHEAS are considered to play a role in memory both in normal subjects and aging patients (Sunderland et al., 1989; Nasman et al., 1991; Strous et al., 2006). More specifically, with regards to Alzheimer's disease, decreased levels of several neurosteroids have been observed in the frontal cortex, hippocampus, amygdala, striatum, hypothalamus, and cerebellum (Sunderland et al., 1989; Nasman et al., 1991; Weill-Engerer et al., 2002; Schumacher et al., 2003). The best known role of neurosteroids is their involvement in the control of mood disorders. Numerous behavioral investigations have shown that neurosteroids exert anxiolytic (Hodge et al., 2002; Strous et al., 2003), anti-depressant (Uzunova et al., 2003; van Broekhoven and Verkes, 2003), anti-aggressive (Kavaliers and Kinsella, 1995; Pinna et al., 2008), hypnotic (Lancel et al., 1997; Damianisch et al., 2001), anti-convulsive (Landgren et al., 1987; Belelli et al., 1989), and anti-stress actions (Patchev et al., 1996; Hu et al., 2000). In animal models, an effect of neurosteroids in eating disorders has been reported (Reddy and Kulkarni, 1998, 1999; Strous et al., 2006). In particular, DHEA decreases food intake (Svec and Porter, 1996; Svec et al., 1998), reduces insulin resistance (Svec and Porter, 1998a,b), and lowers adiposity (Svec and Porter, 1998a,b; Pham et al., 2000). DHEAS has also been found to induce a significant decrease in food intake and body weight (Reddy and Kulkarni, 1999; Kaur and Kulkarni, 2001). In contrast, allopregnanolone causes hyperphagia in male and female rats (Reddy and Kulkarni, 1999). The dose-dependent effects of Δ^5 PS on locomotion of mice placed in a novel environment show the existence of a possible role of neurosteroids in adaptation to novelty (Fahey et al., 1995a). Neurosteroids are also involved in the regulation of excitotoxic and apoptotic processes (Kimonides et al., 1999; Frank and Sagratella, 2000; Manji et al., 2003; Charalampopoulos et al., 2004; Wojtal et al., 2006). *In vivo* studies indicate that progesterone, allopregnanolone, and DHEA exert neuroprotective effects in models of traumatic brain injury (Stein, 2001; Malik et al., 2003; Djebaili et al., 2004; He et al., 2004) and in focal cerebral ischemia (Malik et al., 2003; Sayeed et al., 2006). *In vitro* data also point to the neuroprotective effects of neurosteroids in models of neuronal injury against neurotoxic insults inflicted by excitatory amino acids (Kimonides et al., 1999; Gursoy et al., 2001) and β -amyloid peptides (Kimonides et al., 1998, 1999; Frank and Sagratella, 2000; Lockhart et al., 2002; Xilouri and Papazafiri, 2006). It has been demonstrated that neurosteroids play important functions in neurodevelopment and neuronal remodeling, including neurogenesis (Roberts, 1986; Weaver et al., 1997; Schumacher et al., 2000; Magnaghi et al., 2001; Young, 2002), axonal and dendritic growth, and synaptic connectivity (Jones, 1994; Compagnone and Mellon, 1998). In addition to their protective effects on the brain, neurosteroids can regulate myelin formation and increase expression of myelin proteins in the sciatic nerve (Koenig et al., 1995; Murakami et al., 2000; Plassart-Schiess and Baulieu,

2001; Laurine et al., 2003; Melcangi et al., 2003; Iwata et al., 2005).

The effects of neurosteroids on neuronal activity are mediated through several distinct categories of receptors. Neurosteroids, like other steroid hormones, can act at the transcriptional level through interaction with nuclear receptors (McEwen, 1994; Rupprecht, 1997; Mo et al., 2004). Neurosteroids may also interact with plasma membrane G protein-coupled receptors either directly (Schiess and Partridge, 2005; Tasker et al., 2006) or indirectly by modulating the binding of neuropeptides to their receptors (Grazzini et al., 1998; Zwain et al., 2002; Torres and Ortega, 2003). In addition, neurosteroids can stimulate tubulin polymerization in cultured neurons by binding to the microtubule-associated protein-2 (Murakami et al., 2000; Plassart-Schiess and Baulieu, 2001; Laurine et al., 2003; Iwata et al., 2005). However, most of the actions of neurosteroids are mediated via allosteric modulation of neurotransmitter receptors, including the GABA_A/central-type benzodiazepine receptor (CBR) complex (Belelli and Lambert, 2005; Belelli et al., 2006; Zheng, 2009), *N*-methyl-D-aspartate (NMDA; Mameli et al., 2005; Monnet and Maurice, 2006), kainate (Costa et al., 2000; Dubrovsky, 2005), α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA; Rupprecht and Holsboer, 2001; Dubrovsky, 2005), glycine (Jiang et al., 2006; Mitchell et al., 2007), sigma (Monnet et al., 1995; Maurice et al., 2006), serotonergic (Kostowski and Bienkowski, 1999; Shannon et al., 2005a,b), nicotinic (Valera et al., 1992; Bullock et al., 1997), and muscarinic receptors (Horishita et al., 2005; Steffensen et al., 2006). For example, the effects of GABA on the GABA_A receptor are allosterically potentiated by progesterone and deoxycorticosterone metabolites such as allopregnanolone, pregnanolone, and tetrahydrodeoxycorticosterone (Majewska et al., 1986; Majewska, 1992; Paul and Purdy, 1992; Bergeron et al., 1996). On the other hand, Δ^5 PS and DHEA have been reported to inhibit GABA_A receptor activity (Majewska and Schwartz, 1987; Park-Chung et al., 1999). Allopregnanolone is a negative modulator of the NMDA receptors (Wu et al., 1991; Bowlby, 1993). In contrast, Δ^5 PS and DHEA potentiate several NMDA-receptor-mediated responses, and thus act as excitatory neurosteroids (Wu et al., 1991; Irwin et al., 1992; Fahey et al., 1995b; Compagnone and Mellon, 1998). At the AMPA and kainate receptors, Δ^5 PS behaves as a negative modulator (Rupprecht, 1997; Mellon and Griffin, 2002). It has also been demonstrated that DHEAS acts as a sigma receptor agonist, Δ^5 PS as a sigma receptor inverse agonist, and progesterone as a sigma receptor antagonist (Monnet et al., 1995).

The biochemical pathways leading to the synthesis of steroids in the nervous system of vertebrates have now been almost completely elucidated (Tsutsui and Yamazaki, 1995; Tsutsui et al., 1999; Compagnone and Mellon, 2000; Do Rego et al., 2009; Diotel et al., 2011; Vaudry et al., 2011) and it is now firmly established that neurosteroids regulate neuronal activity (Reddy, 2003, 2010; Dubrovsky, 2005; Belelli et al., 2006; Strous et al., 2006). Over the last decade, a number of studies have thus been undertaken to decipher the neuronal mechanisms involved in the regulation of neurosteroid production. In the present report, we review the current knowledge regarding the effects and mode of action of neurotransmitters, peptide hormones, and neuropeptides on the

biosynthesis of neurosteroids in the CNS of vertebrates. Remarkably, most of these studies have been conducted in the brain of amphibians and birds. In fact, since the rate of steroid synthesis is relatively high in the CNS of frog, quail, and zebra finch (Mensah-Nyagan et al., 1994, 1996; Tsutsui et al., 2006, 2009; Do Rego et al., 2007a; Schlinger and Remage-Healey, 2011), these animals have proved to be suitable models in which to investigate the neuronal mechanisms regulating neurosteroid production.

EFFECT OF GLUTAMATE ON NEUROSTEROID BIOSYNTHESIS

In the quail forebrain, all classes of ionotropic glutamate receptors, i.e., NMDA, AMPA, and kainate receptors, are expressed in the medial preoptic nucleus, a region which is enriched with P450arom-containing cell bodies (Cornil et al., 2000), suggesting that these neurons may be regulated by glutamate. Consistent with this hypothesis, incubation of quail hypothalamic fragments with AMPA and kainate, and to a certain extent NMDA, inhibits P450arom activity (Balthazart et al., 2001a). In mice, administration of NMDA or glutamate within the nidopallium provokes a substantial decrease in the local concentration of estradiol (Remage-Healey et al., 2008; Cornil, 2009). These observations indicate that, in birds and mammals, glutamate exerts an inhibitory action on the formation of neuroestrogens. In the quail brain, the inhibitory effects of AMPA and kainate are mimicked by ATP, Ca^{2+} and Mg^{2+} , and the Ca^{2+} response is abrogated by PKA, PKC, and CAMK (Balthazart et al., 2001b, 2003). Since P450arom possesses several consensus phosphorylation sites in its sequence (Harada, 1988; Balthazart et al., 2003), it appears that the rapid inhibitory effect of AMPA, kainate, or NMDA on P450arom activity can be ascribed to Ca^{2+} -dependent phosphorylation of the P450arom protein (Balthazart et al., 2001c, 2003).

EFFECT OF MELATONIN ON NEUROSTEROID BIOSYNTHESIS

Measurement of neurosteroid content and/or biosynthesis in the brain of vertebrates has revealed that marked changes in $\Delta^5\text{P}$, 7α -hydroxypregnenolone (7α -OH- $\Delta^5\text{P}$), P, THP, and $\Delta^5\text{PS}$ occur during circadian and seasonal cycles (Jo et al., 1990; Takase et al., 1999; Inai et al., 2003; Matsunaga et al., 2004; Tsutsui et al., 2008). These changes often parallel or mirror variations in plasma melatonin concentrations (Hau and Gwinner, 1994; Warren and Cassone, 1995; Marumoto et al., 1996; Murakami et al., 2001). It has been shown that melatonin regulates locomotor activity in house sparrow, Japanese quail, red-bellied newt, ural owl, rock pigeon, and Sprague Dawley rat (Hau and Gwinner, 1994; Warren and Cassone, 1995; Murakami et al., 2001). 7α -OH- $\Delta^5\text{P}$, in very much the same as melatonin, regulates locomotor activity in Japanese quail and red-bellied newt (Matsunaga et al., 2004; Tsutsui et al., 2008), suggesting that melatonin may control 7α -OH- $\Delta^5\text{P}$ production. In support of this notion, male quails which exhibit marked diurnal variations in locomotor activity also show substantial changes in brain level of 7α -OH- $\Delta^5\text{P}$ while female quails which do not show locomotor rhythms have low level of 7α -OH- $\Delta^5\text{P}$ (Tsutsui et al., 2008). Similarly, it has been found that the synthesis of 7α -OH- $\Delta^5\text{P}$ in the brain of male newts undergoes marked diurnal changes with higher levels during the dark phase when locomotor activity of males is high. In contrast, 7α -OH- $\Delta^5\text{P}$ production in female newts does not change (Haraguchi et al., 2009, 2010;

Koyama et al., 2009; Tsutsui et al., 2010). In male quail, suppression of endogenous melatonin through pinealectomy (Px) and orbital enucleation (Ex) causes a significant increase of the expression of CYP7B mRNA and biosynthesis of 7α -OH- $\Delta^5\text{P}$ (Tsutsui et al., 2008). ICV injection of melatonin suppresses the effects of Px and Ex on CYP7B gene transcription and 7α -OH- $\Delta^5\text{P}$ production. Finally, the melatonin receptor antagonist luzindole abrogates the inhibitory effect of melatonin on 7α -OH- $\Delta^5\text{P}$ formation (Tsutsui et al., 2008). Collectively, these data support the contention that, in male birds, melatonin inhibits the expression of CYP7B and that the subsequent decrease of 7α -OH- $\Delta^5\text{P}$ in the brain is responsible for the nocturnal reduction of locomotor activity.

EFFECT OF PROLACTIN ON NEUROSTEROID BIOSYNTHESIS

In male newts, as in many other wild animals, locomotor activity increases during the breeding season (Iwata et al., 2000). This hyperlocomotor response is associated with a concomitant increase in 7α -OH- $\Delta^5\text{P}$ in the newt brain (Haraguchi et al., 2009, 2010). The pituitary hormone prolactin (PRL) exerts pleiotropic functions in the control of reproduction in urodeles (Polzonetti-Magni et al., 1995; Kikuyama et al., 2003). In particular, PRL is involved in migration to water at the breeding season (Chadwick, 1941) and stimulates expression of courtship behavior with rapid tail vibration by male newts (Toyoda et al., 1993). Indeed, in male newts, plasma PRL concentration increases during the breeding season (Matsuda et al., 1990; Mosconi et al., 1994) and it has been shown that PRL acts centrally to activate courtship behavior (Toyoda et al., 2005), suggesting that PRL may be involved in the control of 7α -OH- $\Delta^5\text{P}$ synthesis in the brain to increase locomotor activity during the reproductive period. In support of this hypothesis, PRL receptor immunoreactivity is observed in CYP7B-expressing neurons in the anterior preoptic area (Poa) and the magnocellular preoptic nucleus (Mg) of the newt brain (Haraguchi et al., 2010). Hypophysectomy markedly reduces brain concentration and biosynthesis of 7α -OH- $\Delta^5\text{P}$ in breeding male newts and these effects are suppressed by ICV injection of PRL (Haraguchi et al., 2010). Reciprocally, ICV administration of newt PRL antiserum dose-dependently decreases 7α -OH- $\Delta^5\text{P}$ synthesis (Haraguchi et al., 2010). Taken together, these observations indicate that PRL directly acts on Mg neurons expressing CYP7B to enhance the biosynthesis of 7α -OH- $\Delta^5\text{P}$ which in turn mediates the stimulatory effect of PRL on locomotion.

EFFECT OF GABA ON NEUROSTEROID BIOSYNTHESIS

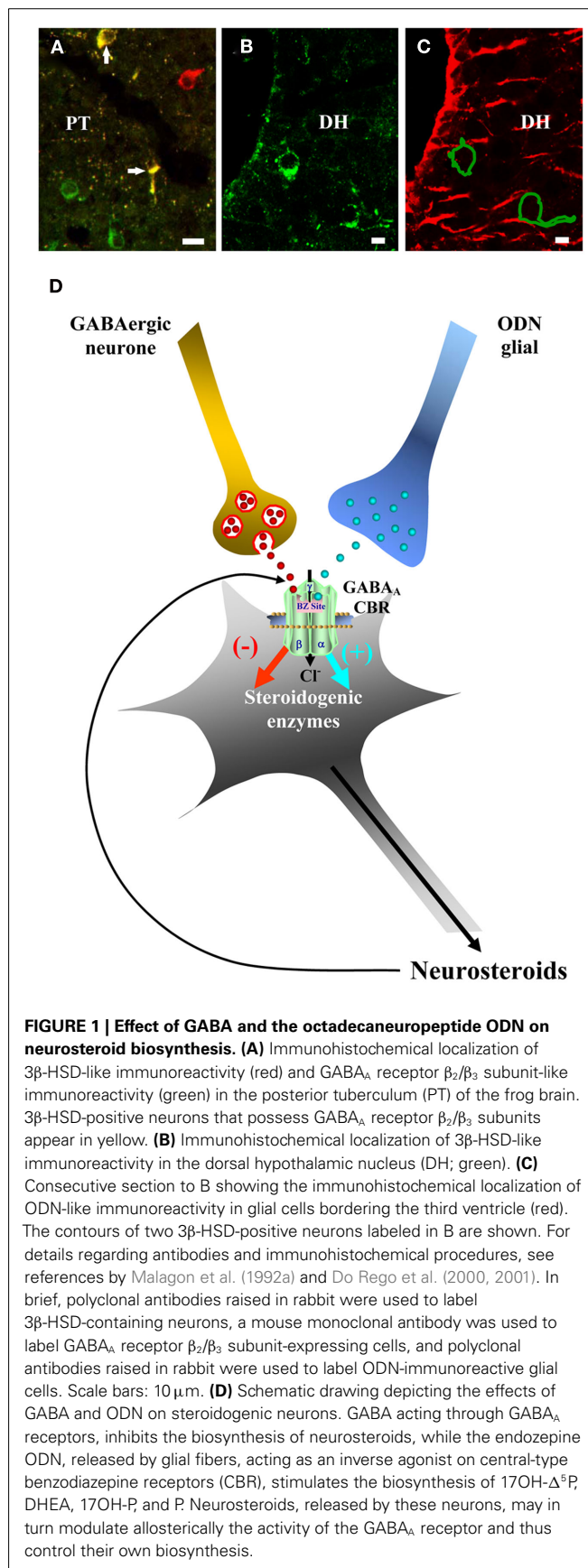
Gamma-aminobutyric acid (GABA) is the major neurotransmitter in the CNS (Krnjevic and Schwartz, 1966; Meldrum, 1982; Paredes and Agmo, 1992). In mammals, brain nuclei that express steroidogenic enzymes (Tsutsui et al., 1999; Do Rego et al., 2009) are innervated by GABAergic nerve fibers (Tappaz et al., 1983; Sakaue et al., 1988) and are also enriched with GABA_A receptors (De Montis et al., 1981; McDonald and Mascagni, 1996; Bäckberg et al., 2004), suggesting that GABA may regulate the activity of steroidogenic nerve cells. As a matter of fact, pharmacological studies have shown that administration of the GABA synthesis inhibitor isoniazid induces an increase of endogenous $\Delta^5\text{P}$ and P in the rat brain (Barbaccia et al., 1996). In contrast, in rat retinal

ganglion cells, GABA, acting through GABA_A receptors, stimulates the biosynthesis of Δ^5 P (Guarneri et al., 1995).

The effect and mechanism of action of GABA in the control of neurosteroidogenesis has been mainly investigated in non-mammalian vertebrate models. Indeed, in amphibians as in mammals, a rich GABAergic innervation (Franzoni and Morino, 1989; Hollis and Boyd, 2005) and a dense accumulation of GABA_A receptor subunits (Aller et al., 1997) have been observed in hypothalamic regions which contain steroidogenic neurons (Mensah-Nyagan et al., 1994, 1999; Tsutsui et al., 1999; Do Rego et al., 2009). In the frog *Rana esculenta*, double labeling experiments have shown the presence of GABA_A receptor $\alpha 3$ and β_2/β_3 subunit-like immunoreactivity in 3β -HSD-expressing cell bodies (Figure 1A) in the Poa, the posterior tuberculum, the nucleus of the periventricular organ, and the ventral and dorsal hypothalamic nuclei of the hypothalamus (Do Rego et al., 2000) suggesting that, in amphibians as in mammals, GABA may play a role in the control of neurosteroid biosynthesis. In agreement with this hypothesis, GABA has been shown to inhibit in a dose-dependent manner *de novo* biosynthesis of various neurosteroids including 17OH- Δ^5 P, P, 17OH-P, and DHEA, by frog hypothalamic explants (Do Rego et al., 2000). The inhibitory effect of GABA on neurosteroid production is mimicked by the GABA_A receptor agonist muscimol and is blocked by the selective GABA_A receptor antagonists bicuculline and SR95531, but is not affected by the GABA_B receptor agonist baclofen (Do Rego et al., 2000). The observation that bicuculline and SR95531 induce on their own a significant stimulation of steroid formation suggests that endogenous GABA exerts a tonic inhibitory control on neurosteroid-producing neurons (Do Rego et al., 2000). These data indicate that GABA inhibits the biosynthesis of neurosteroids through activation of GABA_A receptors (Figure 1D). Since several neuroactive steroids are potent allosteric regulators of GABA_A receptor function in amphibians (Le Foll et al., 1997a,b; Hollis et al., 2004) as in mammals (Majewska, 1992; Belelli and Lambert, 2005; Belelli et al., 2006), these observations reveal the existence of an ultrashort feedback loop through which certain neurosteroids may regulate their own production via modulation of GABA_A receptor activity (Figure 1D).

EFFECT OF ENDOZEPINES ON NEUROSTEROID BIOSYNTHESIS

The term endozepines designates a family of endogenous peptides that act as natural ligands of CBRs and peripheral-type benzodiazepine receptors also called translocator protein (TSPO; Tonon et al., 2006). The occurrence of endozepines has been reported in the CNS of all classes of vertebrates including fish (Malagon et al., 1992b; Matsuda et al., 2007), amphibians (Malagon et al., 1992a; Lihmann et al., 1994), birds (Todaro et al., 1991; Rose et al., 1992), and mammals (Alho et al., 1989; Tonon et al., 1990; Tong et al., 1991; Malagon et al., 1993). The endozepine family encompasses diazepam-binding inhibitor (DBI), an 86-amino acid polypeptide (Guidotti et al., 1983), and its processing products the triakontatetrapeptide (TTN) and the octadecaneuropeptide (ODN; Ferrero et al., 1984; Slobodyansky et al., 1989). TTN is a selective ligand of TSPO (Slobodyansky et al., 1989; Papadopoulos et al., 2006) while ODN acts as an inverse agonist of CBRs



(Ferrero et al., 1984). Endozepines have been shown to regulate steroid secretion by adrenocortical cells (Yanagibashi et al., 1989; Papadopoulos, 1993; Lesouhaitier et al., 1996, 1998) and Leydig cells (Papadopoulos et al., 1990, 1991a,b; Garnier et al., 1993, 1994; Duparc et al., 2003). Concurrently, it has been found that TTN stimulates $\Delta^5\text{P}$ biosynthesis by isolated mitochondria from C6 glioma cells (Papadopoulos et al., 1992) indicating that endozepines may be involved in the regulation of neurosteroid production.

The distribution of endozepines has been investigated in the brain of mammals and amphibians by *in situ* hybridization and immunohistochemistry. In the rat brain, DBI mRNA is expressed in various glial cell populations of the ependyma, area postrema, and cerebellum (Alho et al., 1988; Tong et al., 1991; Bürgi et al., 1999). In rat, monkey, and human, endozepine immunoreactivity is found in astrocytes in the arcuate nucleus of the hypothalamus, ependymocytes bordering the third ventricle, tanycytes in the median eminence, pituicytes in the neurohypophysis, and Bergman cells in the cerebellum (Slobodyansky et al., 1992; Malagon et al., 1993; Alho et al., 1995; Yanase et al., 2002). In the frog *R. esculenta*, DBI mRNA and ODN-like immunoreactivity are present in radial glial cells of the periventricular systems of the diencephalon and rhombencephalon (Malagon et al., 1992a; Lihrmann et al., 1994). Immunolabeling of consecutive sections revealed that, in the Poa, the dorsal and ventral hypothalamic nuclei, the nucleus of the periventricular organ, and the posterior tuberculum of the frog brain, ODN-immunoreactive ependymocytes project toward $3\beta\text{-HSD}$ -positive perikarya (Do Rego et al., 2001; **Figures 1B,C**). These neuroanatomical observations led us to investigate the possible effects of endozepines on neurosteroid biosynthesis in the diencephalon of amphibians.

Incubation of frog hypothalamic slices with graded concentrations of human and rat ODN induces a dose-dependent stimulation of the conversion of $[^3\text{H}]\Delta^5\text{P}$ into $\Delta^4\text{-3-ketosteroids}$ and $\Delta^5\text{-}3\beta\text{-hydroxysteroids}$ *in vitro* (Do Rego et al., 2001). The biological activity of ODN is borne by the C-terminal portion of the peptide (Do Rego et al., 2007b) whose sequence has been strongly preserved across vertebrate species (Mocchetti et al., 1986; Lihrmann et al., 1994; Tonon et al., 2006). The stimulatory action of ODN on neurosteroid biosynthesis is mimicked by $\beta\text{-carbolines}$ that act as inverse agonists of CBR, and is blocked by the CBR antagonist flumazenil (Do Rego et al., 2001). The fact that flumazenil alone inhibits the formation of neurosteroids supports the view that ODN plays a physiological role as an endogenous positive modulator of CBR (Do Rego et al., 2001; **Figure 1D**).

In intact mammalian endocrine cells, TTN stimulates the secretion of glucocorticoids (Yanagibashi et al., 1989; Papadopoulos et al., 1991a) and testosterone (Garnier et al., 1993; Duparc et al., 2003). TTN also stimulates steroidogenesis by mitochondria isolated from adrenocortical and testicular Leydig cells (Besman et al., 1989; Yanagibashi et al., 1989; Papadopoulos et al., 1991a). In frog adrenal tissue, TTN stimulates corticosterone and aldosterone secretion *in vitro* (Lesouhaitier et al., 1996, 1998). Since TSPO is expressed not only in peripheral organs but also in the CNS (Braestrup and Squires, 1977; Benavides et al., 1983a,b; Anholt et al., 1984; Richards and Möhler, 1984; Gehlert et al., 1985), these observations suggest that TTN may be involved in the regulation

of neurosteroidogenesis. To test this hypothesis, we have investigated the effect of TTN on the biosynthesis of steroids in the brain of amphibians.

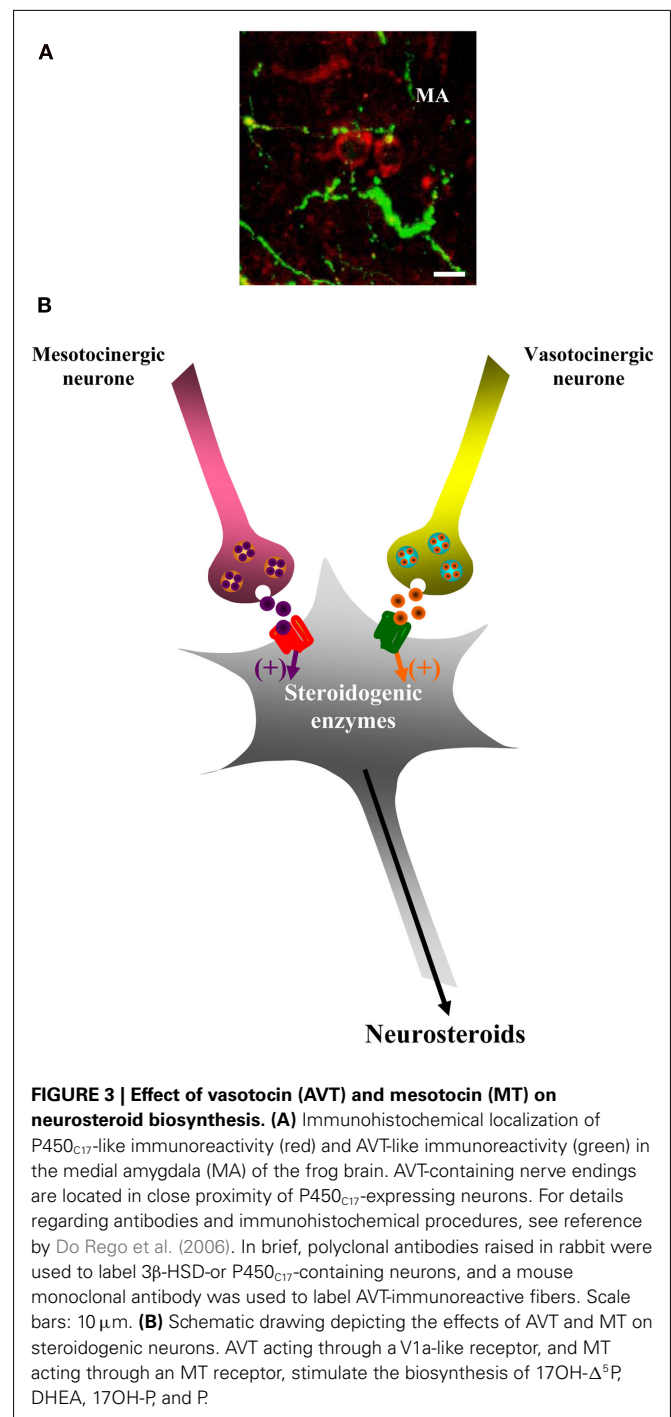
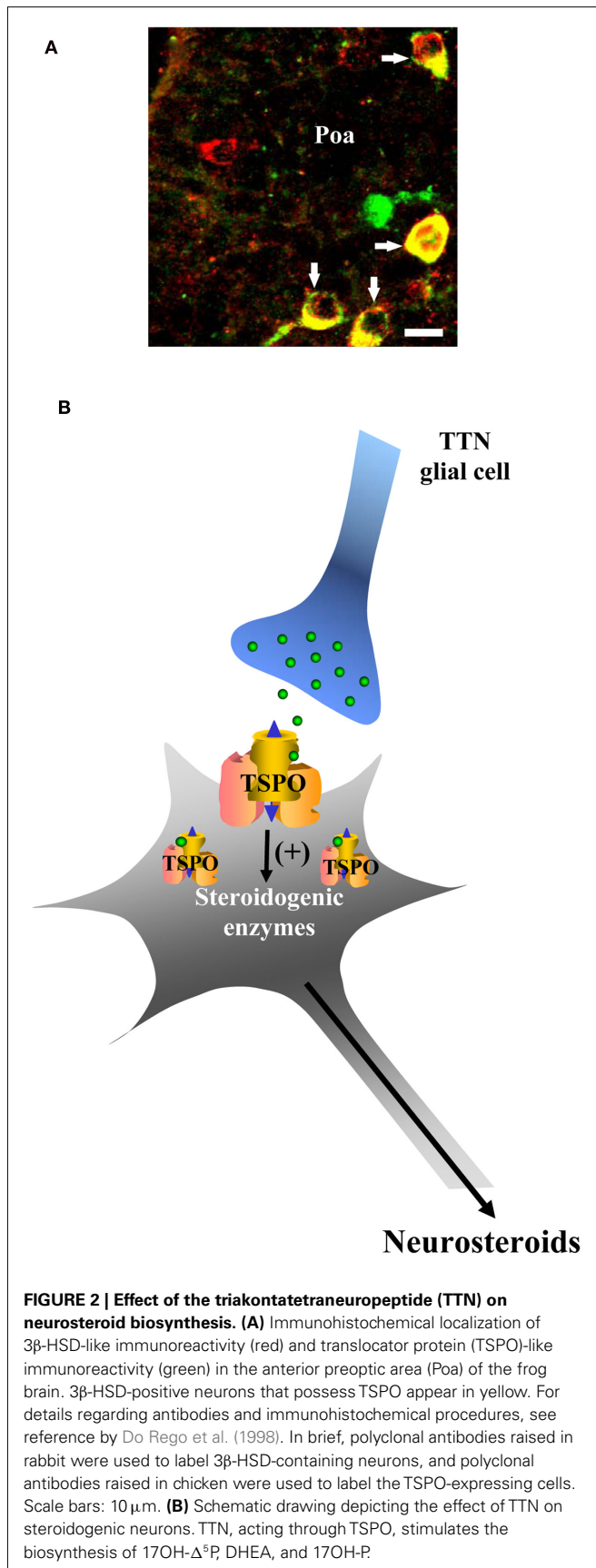
Double labeling experiments have shown the presence of TSPO-like immunoreactivity in steroidogenic neurons of the frog Poa and dorsal hypothalamus (Do Rego et al., 1998; **Figure 2A**). Exposure of frog hypothalamic fragments to synthetic human TTN causes a concentration-dependent stimulation of the conversion of $[^3\text{H}]\Delta^5\text{P}$ into $17\text{OH-}\Delta^5\text{P}$ and 17OH-P (Do Rego et al., 1998). The TSPO agonist Ro5-4864 mimics the stimulatory effect of TTN while the TSPO antagonist PK11195 inhibits TTN-induced steroid production. In contrast, the action of TTN is not impaired by flumazenil, indicating that TTN acts specifically through TSPO to activate neurosteroid biosynthesis (Do Rego et al., 1998; **Figure 2B**).

The fact that ODN and TTN, acting via CBR and TSPO, respectively, stimulate the formation of a number of neurosteroids suggests that endozepines should play multiple functions. Indeed, *in vivo* studies indicate that endozepines, like neurosteroids, exert numerous behavioral effects. In particular, endozepines induce anxiety (Guidotti, 1991; Garcia de Mateos-Verchere et al., 1999), increase aggressivity (Guidotti et al., 1983; Ferrero et al., 1984; Kavaliers and Hirst, 1986; Guidotti, 1991), and possess anti-convulsant properties (Garcia de Mateos-Verchere et al., 1999). In addition, endozepines are potent anorexigenic neuropeptides (Garcia de Mateos-Verchere et al., 2001; Do Rego et al., 2007). Since endozepines and neurosteroids are involved in the control of the same behavioral processes, it is conceivable that neurosteroids can mediate some of the neurobiological effects of endozepines.

EFFECTS OF VASOTOCIN AND MESOTOCIN ON NEUROSTEROID BIOSYNTHESIS

Anatomical studies support the existence of neurochemical communication between steroidogenic neurons on the one hand, and neurons producing either arginine vasopressin (AVP) and oxytocin (OXT) in mammals or their orthologs arginine vasotocin (AVT) and mesotocin (MT) in submammalian vertebrates on the other hand. For instance, in birds, AVT-immunoreactive fibers innervate aromatase-expressing neurons in the preoptic nucleus and the lateral septum (Viglietti-Panzica et al., 1994; Balthazart, 1997). In the brain of amphibians, AVT- and/or MT-containing fibers project in the vicinity of $3\beta\text{-HSD}$ - and P450_{C17} -positive neurons (Do Rego et al., 2006; **Figure 3A**). In addition, the Poa, the dorsal and ventral hypothalamic nuclei, the suprachiasmatic nucleus, the posterior tuberculum, the nucleus of the periventricular organ, and the ventral part of the magnocellular preoptic nucleus of the frog diencephalon that contain the major populations of steroidogenic neurons are also enriched with AVP and MT receptor mRNAs. These close neuroanatomical relationships led us to investigate the possible effects of AVT and MT on neurosteroid biosynthesis in amphibians.

Static incubation of frog hypothalamic explants with AVT or MT stimulates the biosynthesis of $17\text{OH-}\Delta^5\text{P}$, P , 17OH-P , and DHEA in a concentration-dependent manner (Do Rego et al., 2006). This stimulatory effect is observed within the first 30-min, indicating that AVT and MT act at the post-translational level, possibly by activating phosphorylation of steroidogenic enzymes.



The mammalian peptides AVP and OXT also induce a dose-related stimulation of neurosteroid biosynthesis (Do Rego et al., 2006). Pharmacological studies conducted with specific AVT and MT agonists and antagonists revealed that the effect of AVT and MT are mediated through V1a and MT receptors, respectively (Do Rego et al., 2006; **Figure 3B**). These observations are in agreement with the intense expression of V1a and MT receptor mRNAs in the hypothalamic nuclei that contain neurosteroidogenic neurons (Do Rego et al., 2006). Interestingly, in frog adrenocortical cells, AVT

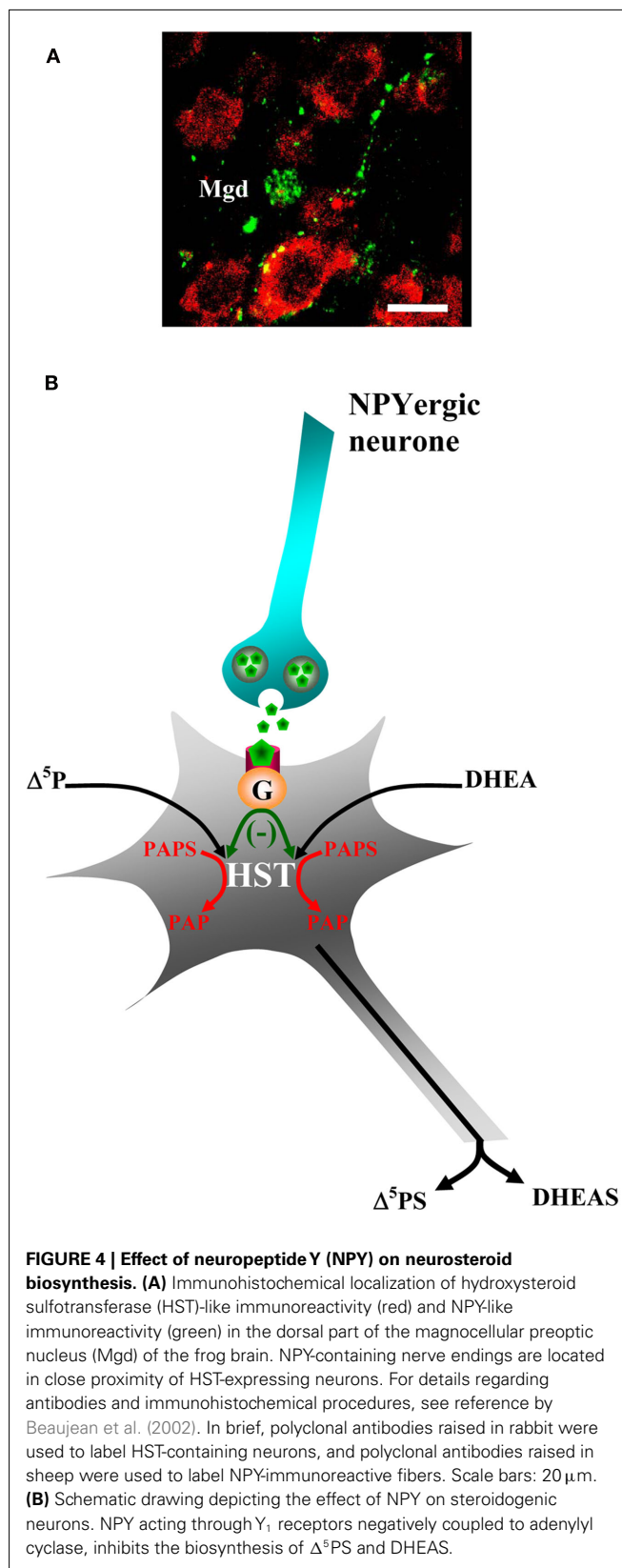
is also a potent stimulator of corticosteroid secretion although, in this latter case, AVT acts via V2 receptors (Larcher et al., 1989, 1992).

In vertebrates, AVP and related peptides exert a vast array of biological effects in the CNS (De Wied et al., 1993; Reghunan-danan et al., 1998). For instance, in mammals, AVP enhances learning and memory (Alescio-Lautier and Soumireu-Mourat, 1998; Croiset et al., 2000; Engelmann et al., 2000; Engelmann, 2008) and increases aggressivity and anxiety-like behaviors (Ferris et al., 1997; Everts and Koolhaas, 1999; Heinrichs and Domes, 2008; Amikishieva et al., 2011; Mak et al., 2011; Meyer-Lindenberg et al., 2011). In birds, depending of the species, AVT increases or attenuates aggressive behavior (Goodson et al., 2004, 2009; Dewan et al., 2011). In amphibians, AVT stimulates vocalization and courtship behavior (Woolley et al., 2004; Moore et al., 2005; Thompson et al., 2008; Kikuyama et al., 2009). Certain neurosteroids also affect cognitive and mnemonic functions, aggressiveness and sexual activity (Frye, 2001; Vallée et al., 2001; Darnaudery et al., 2002; MacKenzie et al., 2007; Morrow, 2007). It thus appears that some of the behavioral effects of AVP/AVT may be ascribed to their stimulatory action on neurosteroid biosynthesis.

EFFECT OF NEUROPEPTIDE Y ON NEUROSTEROID BIOSYNTHESIS

Hydroxysteroid sulfotransferase (HST) catalyzes the transfer of a sulfate moiety from 3'-phosphoadenosine 5'-phosphosulfate (PAPS, a universal donor of sulfate radical) on the 3-hydroxyl group of steroids (Klaassen and Boles, 1997; Strott, 2002). While the presence of HST-containing neurons has been clearly demonstrated in the amphibian hypothalamus (Beaujean et al., 1999; Vaudry et al., 2011), the existence of HST in the brain of mammals is still a matter of debate (Geese and Raftogianis, 2001; Shimada et al., 2001; Shimizu et al., 2003; Kohjitani et al., 2006). Neuropeptide Y (NPY) is one of the most widely distributed biologically active peptides in the CNS of vertebrates (Allen et al., 1983; De Quidt and Emson, 1986; Bons et al., 1990; Danger et al., 1990, 1991; Aste et al., 1991; Hendry, 1993) and NPY, like sulfated neurosteroids, exerts a wide range of neurobiological activities (Dumont and Quirion, 2006).

In frog, the Poa and the magnocellular preoptic nucleus where HST-positive cell bodies are located (Beaujean et al., 1999; Do Rego et al., 2009) are abundantly innervated by NPY-immunoreactive fibers (Danger et al., 1985), suggesting that NPY could play a role in the regulation of HST-expressing neurons. In support of this hypothesis, double labeling experiments revealed that, in the frog diencephalon, numerous NPYergic axon terminals are apposed onto HST-containing perikarya (Beaujean et al., 2002; **Figure 4A**). In addition, the mRNAs encoding the Y₁ and Y₅ NPY receptor subtypes are expressed in the Poa and the magnocellular preoptic nucleus (Beaujean et al., 2002). Incubation of frog hypothalamic tissue with [³H]Δ⁵P or [³H]DHEA as steroid precursors and [³⁵S]PAPS as a sulfate donor leads to the biosynthesis of [³H, ³⁵S]Δ⁵PS or [³H, ³⁵S]DHEAS, respectively, confirming the existence of a biologically active form of HST in the amphibian brain (Beaujean et al., 1999). Synthetic frog NPY (Chartrel et al., 1991) causes a concentration-dependent inhibition of the *de novo* formation of Δ⁵PS and DHEAS (Beaujean et al., 2002). The



inhibitory effect of NPY is suppressed by the Y₁ receptor antagonist BIBP3226 while the Y₂ receptor antagonist NPY₍₁₃₋₃₆₎ is

totally inactive (Beaujean et al., 2002). The fact that the Y₁ receptor antagonist *per se* slightly increases the biosynthesis of Δ^5 PS and DHEAS reveals that endogenous NPY likely acts as a physiological modulator of the formation of sulfated neurosteroids (Beaujean et al., 2002). These data, together with the observation that Y₁ receptor mRNA is expressed in the Poa and the dorsal magnocellular nucleus, provide strong evidence for a Y₁-mediated inhibitory action of NPY on the biosynthesis of sulfated neurosteroids (Figure 4B).

Neuropeptide Y and sulfated neurosteroids are known to regulate the same behavioral activities including response to novelty (von Horsten et al., 1998; Tasan et al., 2009), feeding and satiety (Kalra and Kalra, 2004a,b; Ramos et al., 2005; Beck, 2006; Ueno et al., 2008), and reproductive behavior (Wehrenberg et al., 1989). NPY and sulfated neurosteroids are also implicated in the physiopathology of cognitive disorders, pain, epilepsy, anxiety, and depression (Engel and Grant, 2001; Rupprecht and Holsboer, 2001; Dumont and Quirion, 2006; Gibbs et al., 2006; Reddy, 2010; Thorsell, 2010; Nguyen et al., 2011). The fact that NPY reduces the biosynthesis of sulfated neurosteroids would thus indicate that some of the neurophysiological activities of NPY could be attributed to inhibition of HST activity.

CONCLUDING REMARKS

Studies conducted over the last decade have shown that the biosynthesis of neurosteroids is finely regulated by neurotransmitters (melatonin, GABA), pituitary hormones (prolactin), and various neuropeptides (ODN, TTN, AVT, MT, NPY). Several projects are currently in progress and there is little doubt that additional factors involved in the control of neurosteroid formation will be identified in the near future. As illustrated in the present report, most of the studies aimed at identifying the neurochemical regulation of neurosteroid biosynthesis have been conducted in non-mammalian models, mainly birds and amphibians. It will be now essential to examine whether the same mechanisms are operating in the brain of mammals and to look for their potential physiopathological significance. Little is currently known regarding the molecular

mechanisms implicated in the regulation of neurosteroid biosynthesis. In particular, it has to be determined whether the neuroendocrine factors controlling neurosteroid production act at the transcriptional level to regulate the expression of the genes encoding the steroidogenic enzymes or at the post-translational level, e.g., to modulate the phosphorylation and thus the activity of these enzymes. The fact that neurosteroids are usually implicated in the same biological processes as the neuroendocrine factors which regulate their biosynthesis strongly suggests that some of the neurophysiological and behavioral activities of these latter factors could be mediated by neurosteroids. To test this hypothesis, it will be necessary to investigate whether specific activities of these regulatory factors are impaired in conditional KO mice lacking selectively expression of steroidogenic enzymes in the brain. The neuroendocrine regulation of neurosteroid biosynthesis by neurotransmitters, neuropeptides, and hormonal factors is currently an emerging theme that will undoubtedly flourish in the years to come.

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