Regulation of Neurosteroid Biosynthesis by Neurotransmitters and Neuropeptides

H. Vaudry¹, J.L. Do Rego¹, D. Beaujean-Burel¹, J. Leprince¹, L. Galas¹, D. Larhammar², R. Fredriksson², V. Luu-The³, G. Pelletier³, M.C. Tonon¹, and C. Delarue¹

Summary

It is now established that the brain has the capability of synthesizing biologically active steroids, termed neurosteroids, that participate in the regulation of various neurophysiological and behavioral processes. However, the neuronal mechanisms regulating the activity of neurosteroid-producing cells have not yet been elucidated. We recently found that, in the frog brain, three enzymes involved in steroid biosynthesis are actively expressed in hypothalamic neurons: 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴ isomerase (3β-HSD), cytochrome P450 17α-hydroxylase/C17,20-lyase (P450 C17) and hydroxysteroid sulfotransferase (HST). Concurrently, we showed that frog hypothalamic explants can convert tritiated pregnenolone ([3H]Δ⁵P) into various bioactive steroids, including 17-hydroxypregnenolone (17OH-Δ⁵P), progesterone (P), 17-hydroxyprogesterone (17OH-P), dehydroepiandrosterone (DHEA), Δ⁵P sulfate (Δ⁵PS) and DHEA sulfate (DHEAS). The hypothalamic nuclei, where the 3β-HSD-, P450 C17- and HST-expressing neurons are located, receive afferent fibers containing a variety of neurotransmitters and neuropeptides. Here, we show that GABA, endozepines and neuropeptide Y (NPY) regulate neurosteroid biosynthesis.

Double immunohistochemical labeling of hypothalamic slices with antisera against 3β-HSD and various subunits of the GABA_A receptor revealed that most 3β-HSD-positive neurons also express the α3 and β2/β3 subunits of the GABA_A receptor. Incubation of hypothalamic explants with graded concentrations of GABA induced a dose-dependent inhibition of the conversion of [3H]Δ⁵P into radioactive metabolites. The effect of GABA on neurosteroid biosynthesis was mimicked by the GABA_A receptor agonist muscimol and was blocked by the selective GABA_A receptor antagonists bicuculline and SR95531. The GABA_A

¹ European Institute for Peptide Research, Laboratory of Cellular and Molecular Neuroendocrinology, INSERM U413, UA CNRS, University of Rouen, 76821 Mont-Saint-Aignan, France
² Department of Neuroscience, Unit of Pharmacology, Uppsala University, 75124 Uppsala, Sweden
³ Laboratory of Molecular Endocrinology and Oncology, Laval University Medical Center, Québec, Canada G1V 4G2.

Kordon et al.
Hormones and the Brain
© Springer-Verlag Berlin Heidelberg 2005
receptor complex encompasses a central-type benzodiazepine receptor (CBR). Thus, we investigated the effect of the endozepine octadecaneuropeptide (ODN), an endogenous ligand of CBR, on neurosteroid biosynthesis. Using an antiserum against human ODN, we observed that ODN-positive glial cells send thick processes in the close vicinity of 3β-HSD-containing neurons. Incubation of hypothalamic explants with synthetic ODN induced a dose-dependent stimulation of the conversion of [3H]Δ5P into various neurosteroids. The β-carbolines β-CCM and DMCM, two inverse agonists of CBR, mimicked the stimulatory effect of ODN on neurosteroid biosynthesis, whereas the CBR antagonist flumazenil significantly reduced the stimulatory responses induced by ODN, β-CCM and DMCM. These data indicate that GABA, acting through GABA_A receptors, inhibits 3β-HSD activity and that ODN, acting as an inverse agonist on the GABA_A/CBR complex, stimulates neurosteroid biosynthesis.

Labeling of brain sections revealed the existence of NPY-immunoreactive varicosities in close proximity to HST-containing perikarya. In situ hybridization studies showed that Y_1 and Y_5 receptor mRNAs are expressed in the anterior preoptic area and the dorsal magnocellular nucleus. Pulse-chase experiments with 35S-labeled 3'-phosphoadenosine 5'-phosphosulfate as a sulfate donor, and [3H]Δ5P or [3H]DHEA as a steroid precursor, demonstrated that NPY inhibits the conversion of Δ5P into Δ5PS and DHEA into DHEAS by hypothalamic explants. The inhibitory effect of NPY on the formation of sulfated neurosteroids was mimicked by PYY, a non-selective NPY receptor agonist, and by [Leu^{31},Pro^{34}]NPY, an agonist for non-Y_2 receptors, and was completely suppressed by the Y_1 receptor antagonist BIBP3226. Conversely, the Y_2 receptor agonist NPY(13-36) and the Y_5 receptor agonist [D-Trp^{32}]NPY did not affect the biosynthesis of Δ5PS and DHEAS. These data indicate that NPY, acting through Y_1 receptors, exerts an inhibitory influence on the biosynthesis of sulfated neurosteroids. The present study provides evidence that, in the brain, neurotransmitters and neuropeptides regulate the activity of neurosteroid-producing neurons. Since neurosteroids have been implicated in the control of a number of behavioral and metabolic activities, these data strongly suggest that some of the neurophysiological effects of neurotransmitters and neuropeptides can be mediated through modulation of neurosteroid biosynthesis.

**Introduction**

Biologically active steroids play an essential role in the development, growth and differentiation of the central nervous system (for review, see McEwen 1994). Owing to their lipophilic nature, steroid hormones secreted by the adrenal gland, testis, ovary and placenta can easily cross the blood-brain barrier. Thus, it has long been assumed that steroids acting on nerve cells in the brain were exclusively produced by endocrine glands. However, pioneering studies
conducted by Baulieu and Robel showed that the concentrations of pregnenolone (Δ⁵P) and dehydroepiandrosterone (DHEA) in the central nervous system (CNS) remained elevated long after adrenalectomy and gonadectomy (Corpéchot et al. 1981, 1983) and that the circadian variations of the levels of these steroids in brain tissue are not synchronized with those of circulating steroids (Robel et al. 1986), suggesting that the brain may be a source of biologically active steroids. Subsequently, immunohistochemical localization of cytochrome P450 side-chain cleavage (P450scc) in the rat brain and the observation that this brain enzyme was capable of converting cholesterol into Δ⁵P (Le Goascogne et al. 1987) confirmed that the brain was a steroidogenic organ. The presence of most of the enzymes involved in the biosynthetic pathways of steroids has now been demonstrated, either in neurons or in glial cells, by immunohistochemical or in situ hybridization approaches. These include: P450scc, a desmolase that cleaves the C₂₀–C₂₂ bond of cholesterol; 3β-hydroxysteroid dehydrogenase/Δ⁵-Δ⁴ isomerase (3β-HSD), which catalyzes the formation of Δ⁴-3-ketosteroids; cytochrome P450 17α-hydroxylase/C17,20-lyase (P450c₁₇), which catalyzes the production of Δ⁵-3β-hydroxysteroids; 17β-hydroxysteroid dehydrogenase (17β-HSD), which catalyzes the interconversion of 17-ketosteroids (androstenedione, estrone) and 17β-hydroxysteroids (testosterone, 17β-estradiol); 5α-reductase, which causes the reduction of the C₄-C₅ double bond to produce 5α-reduced metabolites including dihydrotestosterone (5α-DHT) and dihydroprogesterone (5α-DHP); cytochrome P450aromatase, which converts androgens into estrogens; hydroxysteroid sulfotransferase (HST), which is responsible for the synthesis of sulfated steroids such as Δ⁵P sulfate (Δ⁵PS) and DHEA sulfate (DHEAS); and sulfatase, which hydrolyzes sulfated steroids to produce unconjugated steroids (Mensah-Nyagan et al. 1999; Mellon and Vaudry 2001). The term neurosteroids has been coined to designate all biologically active steroids synthesized from cholesterol or other, early precursors in the CNS (Robel and Baulieu 1994).

There is now clear evidence that neurosteroids are involved in the regulation of various neurophysiological and behavioral processes, including cognition, stress, anxiety, sleep, and sexual and feeding behaviors (Majewska 1992; Robel and Baulieu 1994). However, the neuronal mechanisms regulating the activity of neurosteroid-producing cells remain poorly understood. We recently found that, in the frog brain, three enzymes involved in the synthesis of neuroactive steroids - 3β-HSD, P450c₁₇, and HST - are intensely expressed in hypothalamic neurons, and we showed that frog hypothalamic explants can very actively convert tritiated Δ⁵P ([³H]Δ⁵P) into various bioactive steroids, including 17-hydroxypregnenolone (17OH-Δ⁵P), progesterone (P), 17-hydroxyprogesterone (17OH-P), DHEA, Δ⁵PS and DHEAS. The hypothalamic nuclei where 3β-HSD-, P450c₁₇- and HST-expressing neurons are located receive afferent fibers containing a variety of neurotransmitters and neuropeptides. The frog hypothalamus is thus a very suitable model in which to investigate the neuronal control of neurosteroid biosynthesis.
Regulation of neurosteroid synthesis by GABA

In the frog brain, the neurons expressing 3β-HSD and/or P450C17 are exclusively located in the anterior preoptic area and in a few hypothalamic nuclei, including the suprachiasmatic nucleus, the ventral part of the magnocellular preoptic nucleus, the posterior tuberculum, the nucleus of the periventricular organ, the dorsal hypothalamic nucleus and the ventral hypothalamic nucleus (Mensah-Nyagan et al. 1994). Since these diencephalic nuclei are richly innervated by GABAergic fibers (Franzoni and Morino 1989), we investigated the possible effect of GABA in the control of neurosteroid biosynthesis. Double immunohistochemical labeling of hypothalamic slices with antisera against 3β-HSD and various subunits of the GABA_A receptor revealed that many 3β-HSD-positive neurons also express the α3 and the β2/β3 subunits of the GABA_A receptor (Do Rego et al. 2000). Incubation of hypothalamic explants with graded concentrations of GABA induced a dose-dependent inhibition of the conversion of [3H]∆5P into radioactive metabolites, e.g., 17OH-∆5P, P, 17OH-P and DHEA (Do Rego et al. 2000). The inhibitory effect of GABA on neurosteroid biosynthesis was mimicked by the GABA_A receptor agonist muscimol and was

---

Fig. 1. Schematic representation of the effect of GABA and ODN on neurosteroid biosynthesis. GABA, acting through GABA_A receptors, inhibits the biosynthesis of 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone and dehydroepiandrosterone. Concurrently, the endozepine octadecaneuropeptide (ODN), released by glial cell processes ending in the vicinity of 3β-hydroxysteroid dehydrogenase (3β-HSD)-/cytochrome P450C17 (P450C17)-immunoreactive neurons, stimulates the biosynthesis of neurosteroids by acting as an inverse agonist on central-type benzodiazepine receptors (CBR) that are associated with the GABA_A receptor complex. Neurosteroids, released by these neurons, may in turn modulate allosterically the activity of the GABA_A receptor and thus control their own biosynthesis. ∆5P, pregnenolone.
blocked by the selective GABA<sub>A</sub> receptor antagonists bicuculline and SR95531. In contrast, the GABA<sub>B</sub> receptor agonist baclofen did not affect neurosteroid synthesis. Interestingly, bicuculline and SR95531, when administered alone, induced a significant increase in steroid biosynthesis, suggesting that neurosteroid-producing neurons are under the inhibitory control of endogenous GABA (Do Rego et al. 2000). Since neurosteroids are potent allosteric regulators of GABA<sub>A</sub> receptor function (Covey et al. 2001), these data suggest the existence of an ultrashort regulatory feedback loop by which neurosteroids may regulate their own production through modulation of GABA<sub>A</sub> receptor activity (Fig. 1).

**Regulation of neurosteroid synthesis by endozepines**

The activity of the GABA<sub>A</sub> receptor can be allosterically regulated by various compounds, including neuroactive steroids, barbiturates, ethanol, zinc and benzodiazepines (Hevers and Luddens 1998). The existence of specific binding sites for benzodiazepines in the brain has led to the characterization of endogenous ligands for benzodiazepine receptors, a family of molecules termed endozepines. All endozepines identified so far derive from an 86-amino acid polypeptide called diazepam-binding inhibitor (DBI; Guidotti et al. 1983). Proteolytic cleavage of DBI has the potential to generate several biologically active fragments, including the triakontatetraneuropeptide (TTN) and the octadecaneuropeptide (ODN; Fig. 2). While ODN is a preferential ligand for the central-type benzodiazepine receptor (CBR) that belongs to the GABA<sub>A</sub> receptor complex (Ferrero et al. 1986), TTN is a specific ligand for the peripheral-type benzodiazepine receptor (Slobodyansky et al. 1989).

Immunohistochemical labeling of consecutive frog brain slices with antibodies against 3β-HSD and human ODN (Duparc et al. 2003) revealed that ODN-positive periventricular glial cells send thick immunoreactive processes in the close vicinity of 3β-HSD-containing neurons in various hypothalamic nuclei (Do Rego et al 2001). Incubation of hypothalamic explants with synthetic rat or human ODN stimulated, in a dose-dependent manner, the conversion of [3H]Δ5P into various neurosteroids. The β-carbolines β-CCM and DMCM, two inverse agonists of CBRs, mimicked the effect of ODN on neurosteroid biosynthesis, whereas flumazenil, a CBR antagonist, significantly reduced the stimulatory response induced by ODN, β-CCM and DMCM. In fact, flumazenil induced a significant reduction of neurosteroid production on its own, indicating that endogenous ODN exerts a stimulatory influence on steroidogenic neurons (Do Rego et al. 2001). In addition, the ODN-evoked stimulation of steroid synthesis was markedly attenuated by GABA. These data indicate that the inhibitory effect of GABA on neurosteroid biosynthesis can be modulated by ODN, acting as an inverse agonist on the GABA<sub>A</sub>/central-type benzodiazepine receptor complex (Fig. 1).
The occurrence of peripheral-type benzodiazepine receptors in the frog diencephalon and telencephalon has been demonstrated by immunohistochemistry using antibodies against the 18-kDa subunit of peripheral-type benzodiazepine receptors (Do Rego et al. 1998). Double labeling of brain slices with antisera against 3β-HSD and the 18-kDa subunit of peripheral-type benzodiazepine receptors revealed that most hypothalamic neurons expressing 3β-HSD also contained peripheral-type benzodiazepine receptor-like immunoreactivity. Exposure of hypothalamic explants to TTN induced a dose-related increase in the production of 17OH-Δ5P, 17OH-P and a novel neurosteroid that has not yet been identified (Do Rego et al. 1998). The stimulatory effect of TTN on the formation of neurosteroids was mimicked by the peripheral-type benzodiazepine receptor agonist Ro5-4864 and was markedly reduced by the peripheral-type benzodiazepine receptor antagonist PK11195. In contrast, the CBR antagonist flumazenil did not affect the activation of neurosteroid biosynthesis induced by TTN (Do Rego et al. 1998). These data indicate that TTN, acting via peripheral-type benzodiazepine receptors, stimulates the activity of several key steroidogenic enzymes, including 3β-HSD and P450C17.

Fig. 2. Schematic representation of three major endozepines. Diazepam-binding inhibitor (DBI) is an 86-amino acid polypeptide that encompasses a high proportion of basic residues, i.e., Lys (K) or Arg (R). Proteolytic cleavage of DBI at some of these basic amino acids (arrows) can generate several processing products, including the triakontatetraneuropeptide (TTN; DBI17-50) and the octadecaneuropeptide (ODN; DBI33-50). DBI and ODN act as preferential ligands for central-type benzodiazepine receptors (CBRs) that are part of the GABA_A receptor complex, whereas TTN is a specific ligand for peripheral-type benzodiazepine receptors that are primarily located at the outer surface of mitochondria.
Regulation of neurosteroid synthesis by neuropeptide Y

In the frog brain, HST-positive neurons are located in the anterior preoptic area and the dorsal magnocellular nucleus (Beaujean et al. 1999), two diencephalic nuclei that are richly innervated by neuropeptide Y (NPY)-containing fibers (Danger et al. 1985; Caillez et al. 1987; Lázár et al. 1993). Concurrently, there is evidence that sulfated neurosteroids and NPY are involved in the regulation of similar behavioral activities. For instance, Δ⁵PS and DHEAS, like NPY, are implicated in the control of food intake in rodents (Reddy and Kulkarni 1998; Schwartz et al. 2000). Similarly, Δ³PS and NPY are known to regulate reproductive behavior (Wehrenberg et al. 1989; Kavaliers and Kinsella 1995). These observations prompted us to investigate the possible effects of NPY on the biosynthesis of sulfated neurosteroids.

Double labeling of frog brain sections revealed the existence of NPY-immunoreactive varicosities in close proximity to HST-containing perikarya (Beaujean et al. 2002). Partial sequences of frog (Rana esculenta) Y₁, Y₂, Y₅ and y₆ receptor cDNAs have been cloned, and riboprobes have been used to localize the NPY receptor mRNAs in the frog brain. Expression of Y₁ and Y₅ receptor mRNAs was visualized in the anterior preoptic area and the dorsal magnocellular nucleus, i.e., in the two diencephalic nuclei where HST-immunoreactive neurons are located. Neither Y₂ nor y₆ receptor transcripts were found in the frog diencephalon, suggesting that NPY might regulate the activity of HST neurons through activation of Y₁ and/or Y₅ receptors (Beaujean et al. 2002).

The effect of NPY on the biosynthesis of sulfated steroids has been studied by means of a pulse-chase technique using either [³H]Δ⁵P or [³H]DHEA as a steroid precursor and 35S-labeled 3’-phosphoadenosine 5’-phosphosulfate as a sulfate donor. Incubation of diencephalic explants with graded concentrations of frog NPY (Chartrel et al. 1991) induced a dose-dependent inhibition of [³H]Δ⁵PS and [³H]DHEAS formation (Beaujean et al. 2002). The inhibitory effect of NPY on the biosynthesis of sulfated neurosteroids was mimicked by peptide YY (PYY), a nonselective NPY receptor agonist, and by [Leu³¹,Pro³⁴]NPY, an agonist for non-Y₂ receptors. In contrast, the N-terminally truncated NPY analogue NPY(13-36), a selective Y₂ receptor agonist, and [D-Trp³²]NPY, a specific Y₅ receptor agonist, did not significantly affect the production of Δ⁵PS and DHEAS. Moreover, compound BIBP3226, a selective Y₁ receptor antagonist, abolished the effect of NPY on neurosteroid formation. These observations indicate that NPY-induced inhibition of Δ⁵PS and DHEAS production is mediated through the Y₁ receptor subtype. The fact that the Y₁ receptor gene is actively expressed in the anterior preoptic area and dorsal magnocellular nuclei, which contain HST-immunoreactive cell bodies (Beaujean et al. 1999), provides additional support for the involvement of Y₁ receptors in the inhibitory effect of NPY on HST activity in the diencephalon. Concurrently, we observed that the Y₁ receptor antagonist BIBP3226 alone provoked a modest increase in the biosynthesis of
∆⁵PS and DHEAS, suggesting that endogenous NPY may actually exert a tonic inhibitory action on sulfated neurosteroid biosynthesis (Beaujean et al. 2002), (Fig. 3).

### Conclusion

The present report provides evidence that, in the brain, various neurotransmitters and neuropeptides regulate the activity of neurosteroid-producing neurons (Fig. 4). Since neurosteroids have been implicated in the control of a number of behavioral and metabolic activities, such as response to novelty, food consumption, sexual activity, aggressiveness, anxiety, depression, body temperature and blood pressure, these data strongly suggest that some of the neurophysiological effects of GABA, endozepines and NPY (and possibly those of many other neurotransmitters and neuropeptides) can be mediated through modulation of neurosteroid biosynthesis.
Fig. 4. Schematic representation of the control of neurosteroid-producing neurons by neurotransmitters and neuropeptides. Fibers containing various stimulatory or inhibitory factors innervate neurosteroid-producing neurons. Several of these factors have been shown to modulate the biosynthesis of neurosteroids. It is thus conceivable that some of the neurophysiological effects of neurotransmitters and neuropeptides can be mediated through modulation of neurosteroid biosynthesis. GABA, γ-aminobutyric acid; NPY, neuropeptide Y; ODN, octadecaneuropeptide.

Acknowledgments

This work was supported by grants from INSERM (U413), a FRSQ-INSERM exchange program (to G.P. and H.V.), and the Conseil Régional de Haute-Normandie.

References


Guidotti A, Forchetti CM, Corda MG, Konkel D, Bennett CD, Costa E (1983) Isolation, characterization, and purification to homogeneity of an endogenous polypeptide with agonistic action on benzodiazepine receptors. Proc Natl Acad Sci USA 80: 3531-3535


