Structure−Activity Relationships of a Series of Analogs of the Endozepine Octadecaneuropeptide (ODN_{11-18}) on Neurosteroid Biosynthesis by Hypothalamic Explants

Jean Luc Do Rego,[†] Jérôme Leprince,[†] Van Luu-The,[‡] Georges Pelletier,[‡] Marie-Christine Tonon,[†] and Hubert Vaudry*[,†]

INSERM U413, Laboratory of Cellular and Molecular Neuroendocrinology, European Institute for Peptide Research (IFRMP 23), University of Rouen, 76821 Mont-Saint-Aignan, France, and Medical Research Council Group in Molecular Endocrinology and Oncology, Laval University Medical Center, Québec G1V 4G2, Canada

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We have previously shown that the endozepine octadecaneuropeptide (ODN) stimulates the biosynthesis of neurosteroids from frog hypothalamic explants. In the present study, we have investigated the structure−activity relationships of a series of analogs of the C-terminal octapeptide of ODN (OP) on neurosteroid formation. We found that OP and its cyclic analog $cyclo_{1-8}$OP stimulate in a concentration-dependent manner the synthesis of various steroids including 17-hydroxypregnenolone, progesterone, 17-hydroxyprogesterone and dehydroepiandrosterone. Deletion or Ala-substitution of the Arg^1 or Pro^2 residues of OP did not affect the activity of the peptide. In contrast, deletion or replacement of any of the amino acids of the C-terminal hexapeptide fragment totally abolished the effect of OP on neurosteroid biosynthesis. The present study indicates that the C-terminal hexapeptide of ODN/OP is the minimal sequence retaining full biological activity on steroid-producing neurons.

Introduction

Endozepines are a family of regulatory peptides that have been originally identified on the basis of their ability to displace diazepam from its binding sites.[1] All endozepines characterized so far derive from an 86-amino acid polypeptide called diazepam-binding inhibitor (DBI[a]) that, like β-carboline, acts as an inverse agonist of central-type benzodiazepine receptors (CBR).[2] Proteolytic cleavage of DBI has the potential to generate several biologically active peptides including the triakontatetraneuropeptide DBI_{17-50} (TTN) and the octadecaneuropeptide DBI_{33-50} (ODN)[3−6] (Figure 1). The effects of DBI and ODN are mediated through CBR associated with the $GABA_A$ receptor complex,[3,7] while TTN acts as a selective ligand of peripheral-type benzodiazepine receptors (PBR).[5,8] In addition, it has been demonstrated that ODN can activate a membrane receptor positively coupled to phospholipase C through a PTX-sensitive G-protein.[9,10]

Endozepines are widely distributed in the central nervous system (CNS) and in various peripheral organs.[2,11−16] Consistent with their broad distribution, endozepines have been found to exert a large array of biological activities such as inhibition of glucose-induced insulin release,[11,17] stimulation of cholecystokinin secretion,[18] activation of glial cell proliferation,[19] and stimulation of steroid hormone biosynthesis.[20−23] However, little is currently known regarding the structure−activity relationships of endozepines. It has just been shown that the C-terminal octapeptide (OP) can displace tritiated β-carboline binding from brain membrane receptors[3] and stimulate intracellular calcium mobilization in cultured rat astrocytes.[24]

Neuroactive steroids can mimic several of the behavioral and neurophysiological effects of endozepines.[2,25−27] Most of the enzymes involved in steroid biosynthesis, including cytochrome P450scc (P450scc), cytochrome $P450_{C17}$ ($P450_{C17}$), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD), aromatase, 5α-reductase, and sulfotransferase, have now been localized in neurons and/or in glial cells in the CNS.[28−31] In particular, it has been found that frog hypothalamic neurons express 3β-HSD, a key enzyme of the steroid biosynthetic pathway, and it has been demonstrated that frog hypothalamic tissue can convert the steroid precursor pregnenolone (Δ^5P) into various bioactive metabolites.[32−34] We have recently found that, in the frog diencephalon, several populations of ODN-immunoreactive glial cells send their thick processes in the close vicinity of 3β-HSD-expressing neurons.[35] We have also shown that ODN, acting through CBR, stimulates the biosynthesis of Δ^4-3-keto- and Δ^5-3β-hydroxy-neurosteroids, suggesting that the endozepine ODN acts as a paracrine factor regulating the activity of 3β-HSD and $P450_{C17}$ cells.[35]

The aim of the present study was to investigate the structure−activity relationships of a series of OP analogs on steroid biosynthesis by frog hypothalamic explants to identify the minimum bioactive sequence of ODN stimulating 3β-HSD and $P450_{C17}$ activity. We have also characterized the pharmacologi-

* To whom correspondence should be addressed. Phone: +33 235 146624. Fax: +33 235 146946. E-mail: hubert.vaudry@univ-rouen.fr.
† INSERM U413, European Institute for Peptide Research.
‡ Laval University Medical Center.

[a] Abbreviations: Symbols and abbreviations are in accord with the recommendations of the IUPAC-IUB Joint Commission on Biochemical Nomenclature: Nomenclature and Symbolism for Amino Acid and Peptides, *Biochem. J.* **1984,** *219*, 345−373; A Short Guide to Abbreviations and Their Use in Peptide Science. *J. Peptide Sci.* **2003**, *9*, 1−8. Additional abbreviations are as follows: ANOVA, analysis of variance; Boc, *t*-butyloxycarbonyl; BSA, bovine serum albumin; CBR, central-type benzodiazepine receptors; CNS, central nervous system; DBI, diazepam-binding inhibitor; Δ^5P, pregnenolone; [^{3}H]Δ^5P, tritiated pregnenolone; DHEA, dehydroepiandrosterone; DIEA, diisopropylethylamine; DMF, dimethylformamide; F, cortisol; Flu, flumazenil; GABA, gamma aminobutyric acid; HBTU, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HEPES, *N*-2-hydroxy-ethyl-piperazine-*N*′-2-ethane sulfonic acid; HMP, 4-hydroxymethyl-phenoxymethyl-copolystyrene-1%-divinylbenzene; HOBt, 1-hydroxy-benzotriazole; MALDI-TOF-MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; NMDA, *N*-methyl-D-aspartate; NMP, *N*-methylpyrrolidin-2-one; ODN, octadecaneuropeptide; OP, octapeptide; O*t*-BU, O-*tert*-butyl; P, progesterone; Pbf, pentamethyldihydrobenzofuran; PBR, peripheral-type benzodiazepine receptors; PEG-PS, polyethyleneglycol-polystyrene; $P450_{C17}$, cytochrome P450 17α-hydroxylase / C17, 20-lyase; P450scc, cytochrome P450 side-chain cleavage; PTX, pertussis toxin; RP-HPLC, reversed-phase high-performance liquid chromatography; S, 11-deoxycortisol; T, testosterone; *t*-BU, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl; TTN, triakontatetraneuropeptide; 3β-HSD, 3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4 isomerase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; 17OH-Δ^5P, 17-hydroxypregnenolone; 17OH-P, 17-hydroxyprogesterone.

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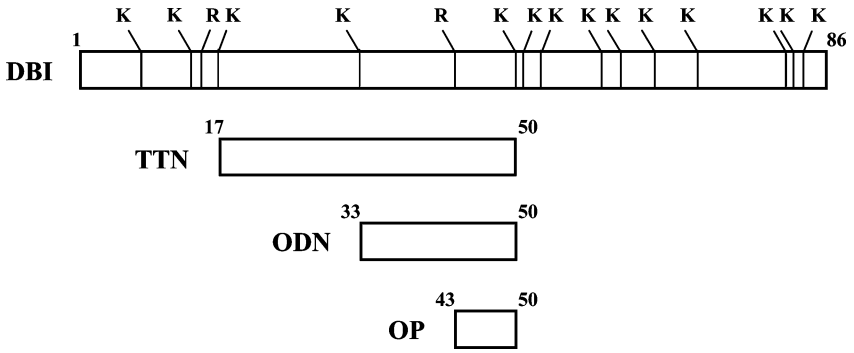

Figure 1. Schematic representation of the amino acid sequence of rat diazepam-binding inhibitor (DBI) and its processing products, that is, the triakontatetraneuropeptide TTN (DBI_{17-50}), the octadecaneuropeptide ODN (DBI_{33-50}) and the octapeptide OP (DBI_{43-50}). Potential mono/polybasic cleavage sites are indicated by vertical bars.

cal profile of the receptor mediating the action of OP and related peptides on neurosteroid production.

Results

Effect of ODN Analogs on Neurosteroid Biosynthesis. After a 2 h incubation of frog hypothalamic explants with tritiated pregnenolone ([^{3}H]Δ^5P), reversed-phase high performance liquid chromatography (RP-HPLC) analysis of the tissue extracts made it possible to resolve several radioactive compounds including 17-hydroxypregnenolone (17OH-Δ^5P), progesterone (P), 17-hydroxyprogesterone (17OH-P), dehydroepiandrosterone (DHEA) and testosterone (T; Figure 2a). Exposure of frog hypothalamic slices to 10^{-7} M OP (ODN_{11-18}; compound **1**, Table 1) provoked a marked increase in the formation of radioactive steroids (Figure 2b). Incubation of hypothalamic slices with graded concentrations of compound **1** or its cyclic analog $cyclo_{1-8}$OP (compound **2**, Table 1; 10^{-10} to 10^{-5} M) induced a concentration-related increase in 17OH-Δ^5P (Figure 3a), P (Figure 3b), 17OH-P (Figure 3c) and DHEA biosynthesis (Figure 3d). The maximum responses were observed at a concentration of 10^{-6} M. At higher concentrations (3×10^{-6} M and 10^{-5} M), the stimulatory effects of compounds **1** and **2** on the formation of radioactive neurosteroids declined. Comparison of the amplitude of the response induced by compounds **1** and **2** with that induced by ODN (compound **3**, Table 1) revealed that, at a concentration of 10^{-6} M, compounds **1** and **2** were less efficient than compound **3** (Figure 3). To determine the minimum biologically active region of compound **1** that was required to stimulate neurosteroidogenesis, a series of short chain (compounds **4**−**12**, Table 1) and L-alanine-substituted analogs (compounds **13**−**20**, Table 1) were synthesized. The C-terminal heptapeptide des-(Arg^1)-OP (compound **4**) and hexapeptide des-(Arg^1, Pro^2)-OP (compound **5**) mimicked the stimulatory effect of compound **1** on the conversion of [^{3}H]Δ^5P into 17OH-Δ^5P and P (Table 2). In contrast, the C-terminal pentapeptide des-(Arg^1, Pro^2, Gly^3)-OP (compound **6**) and the N-terminal heptapeptide des-(Lys^8)-OP (compound **7**), hexapeptide des-(Leu^7, Lys^8)-OP (compound **8**), and pentapeptide des-(Asp^6, Leu^7, Lys^8)-OP (compound **9**), as well as the C- and N-terminally truncated hexapeptide des-(Arg^1, Lys^8)-OP (compound **10**), pentapeptides des-(Arg^1, Pro^2, Lys^8)-OP (compound **11**), and des-(Arg^1, Leu^7, Lys^8)-OP (compound **12**) had no significant effect on neurosteroid biosynthesis (Table 2). The analogs in which the Arg^1 or Pro^2 residues were replaced by an L-Ala moiety (compounds **13** and **14**) retained full biological activity (Table 2). In contrast, Ala substitution of each amino acid of the C-terminal hexapeptide fragment (compounds **15**−**20**) suppressed the neurosteroidogenic activity of compound **1** (Table 2).

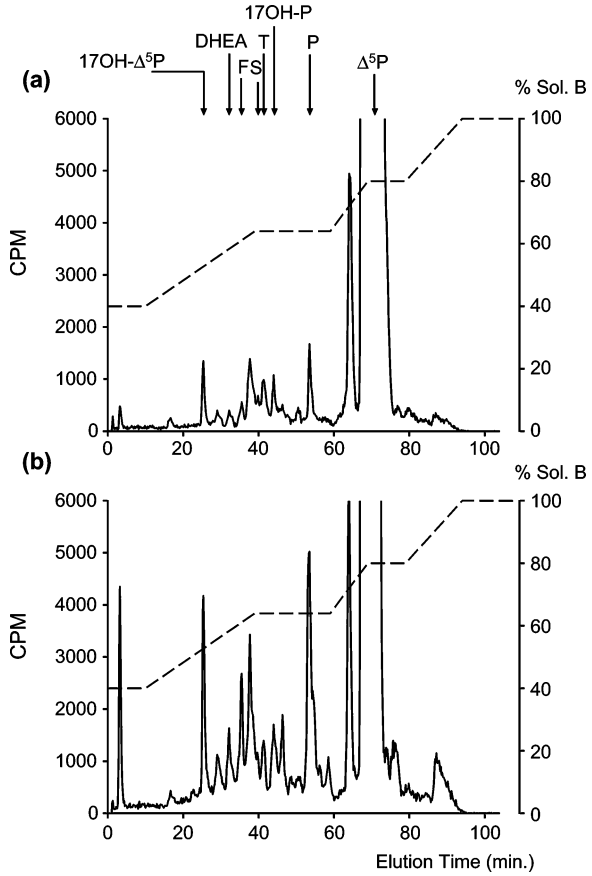

Figure 2. Analysis of radioactive steroids formed after a 2 h incubation of frog hypothalamic explants with [^{3}H]-pregnenolone in the absence (a) or presence of 10^{-7} M OP (compound **1**; b). The ordinate indicates the radioactivity measured in the HPLC eluent. The dashed lines represent the gradient of secondary solvent (% solution B). The arrows indicate the elution positions of standard steroids: 17OH-Δ^5P, 17-hydroxypregnenolone; DHEA, dehydroepiandrosterone; F, cortisol; S, 11-deoxycortisol; T, testosterone; 17OH-P, 17-hydroxyprogesterone; P, progesterone; Δ^5P, pregnenolone.

Receptors Involved in the Stimulatory Effect of OP and OP Analogs on Neurosteroid Biosynthesis. Two selective antagonists were used to characterize the pharmacological profile of the receptor mediating the action of compound **1** and its cyclic analog compound **2** on the biosynthesis of 17OH-Δ^5P, P, 17OH-P, and DHEA (Figures 4 and 5). The CBR antagonist flumazenil (10^{-5} M) totally suppressed the stimulatory effect of compounds **1** (Figure 4) and **2** (Figure 5) on neurosteroid biosynthesis. In addition, flumazenil significantly inhibited the spontaneous conversion of [^{3}H]Δ^5P into 17OH-Δ^5P, P, 17OH-P and DHEA (Figures 4 and 5). In contrast, $cyclo_{1-8}$[DLeu^5]OP (compound

Table 1. Analytical Data on Compounds **1**−**21**

		mass spectrometry		RP-HPLC − 1[a]		RP-HPLC − 2[b]	
No	analog abbreviation	calcd[c]	obsd[d]	R_t (min)	purity (%)	R_t (min)	purity (%)
1	OP	910.56	911.46	16.9	99.9	15.3	99.9
2	cyclo$_{1-8}$OP	892.55	893.35	17.2	98.7	18.3	98.5
3	ODN	1911.01	1912.17	17.6	98.9	18.9	99.1
4	des-(Arg^1)-OP	754.46	755.24	17.9	99.9	15.9	99.9
5	des-(Arg^1, Pro^2)-OP	657.41	658.34	17.5	99.9	11.6	99.9
6	des-(Arg^1, Pro^2, Gly^3)-OP	600.38	601.38	16.3	99.9	6.4	99.9
7	des-(Lys^8)-OP	782.46	783.39	19.4	98.6	17.9	99.1
8	des-(Leu^7, Lys^8)-OP	669.38	670.53	13.8	99.5	4.9	98.7
9	des-(Asp^6, Leu^7, Lys^8)-OP	554.35	555.37	15.1	99.8	5.4	99.6
10	des-(Arg^1, Lys^8)-OP	626.36	627.47	20.5	99.9	18.25	99.9
11	des-(Arg^1, Pro^2, Lys^8)-OP	529.31	530.49	20.0	99.9	15.6	99.9
12	des-(Arg^1, Leu^7, Lys^8)-OP	513.28	514.19	14.6	99.8	5.4	99.8
13	[Ala^1]OP	825.50	826.38	17.5	99.6	16.1	99.4
14	[Ala^2]OP	884.54	885.42	16.9	99.6	12.2	99.7
15	[Ala^3]OP	924.58	925.57	17.1	98.7	15.5	99.2
16	[Ala^4]OP	868.51	869.61	14.2	99.3	5.7	99.6
17	[Ala^5]OP	868.51	869.62	14.3	99.1	5.2	98.8
18	[Ala^6]OP	866.57	867.62	16.5	99.9	13.9	99.9
19	[Ala^7]OP	868.51	869.48	14.3	99.9	4.9	99.9
20	[Ala^8]OP	853.50	854.49	18.5	99.7	17.6	99.9
21	cyclo$_{1-8}$[D-Leu^5]OP	892.55	893.48	19.3	98.2	19.2	98.4

[a] Analytical RP-HPLC analysis, performed on a Vydac 218TPS4 C_{18} column (see Experimental Section). [b] Analytical RP-HPLC analysis, performed on a Vydac 219TPS4 diphenyl column (see Experimental Section). [c] Theoretical monoisotopic molecular weight. [d] The *m*/*z* value assessed by MALDI-TOF−MS.

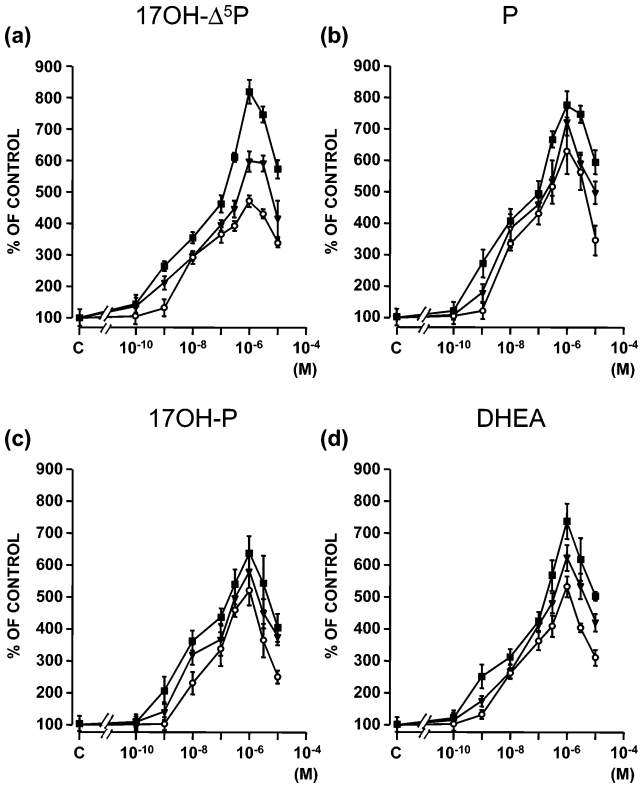

Figure 3. Effects of graded concentrations of OP (compound **1**; ▼), cyclo$_{1-8}$OP (compound **2**; ○), and ODN (compound **3**; ■) on the conversion of [^{3}H]-pregnenolone into (a) 17-hydroxypregnenolone (17OH-Δ^5P), (b) progesterone (P), (c) 17-hydroxyprogesterone (17OH-P), and (d) dehydroepiandrosterone (DHEA) by frog hypothalamic explants. The values were calculated from chromatograms similar to those shown in Figure 2. Results are expressed as percentages of the amount of each steroid formed in the absence of peptide. Values are the mean (±SEM) of four independent experiments.

21, Table 1), a specific antagonist of the metabotropic endozepine receptor,[36] at a concentration of 10^{-5} M, had no effect on the basal production of neurosteroids (Figures 4 and 5 and Table 2) and did not affect the action of compounds **1** (Figure 4) and **2** (Figure 5). Similarly, the stimulatory effect of compound **3** on neurosteroid biosynthesis was not affected by compound **21** (Figure 6).

Discussion

In the rat and frog brain, the DBI gene is expressed in various types of glial cells, notably in radial ependymocytes bordering the third ventricle.[13,14,16,37−39] We have recently observed that, in the frog diencephalon, thick processes originating from ependymal cells contact hypothalamic neurons expressing the steroidogenic enzyme 3β-HSD, and we have shown that the endozepine ODN stimulates neurosteroid biosynthesis.[35] In the present report, we describe the structure−activity relationships of a series of short-chain ODN analogs on the production of Δ^4-3-ketosteroids and Δ^5-3β-hydroxysteroids by frog hypothalamic explants.

The C-terminal ODN octapeptide **1** and its head-to-tail analog **2** stimulated the conversion of the tritiated precursor [^{3}H]Δ^5P into 17OH-Δ^5P, P, 17OH-P, and DHEA in a dose-dependent manner. This observation indicates that compounds **1** and **2** enhance the catalytic activity of at least two steroidogenic enzymes, that is, 3β-HSD and cytochrome P450$_{C17}$. The dose−response effects of compounds **1** and **2** on neurosteroid biosynthesis were very similar to those of compound **3**. In particular, the maximum effective doses of compounds **1**, **2**, and **3** were identical and, at higher concentrations, the responses to all three compounds declined, suggesting the existence of a desensitization mechanism. However, while in frog, compounds **1** and **2** were 1.2 to 3 times less efficacious than compound **3** to stimulate neurosteroid biosynthesis, in cultured rat astrocytes, compound **1** exhibits the same activity as compound **3**, and compound **2** is slightly more efficacious than compound **3** to stimulate inositol phosphate formation and to enhance cytosolic calcium concentration.[24,36]

Structure−activity relationship studies, using a series of truncated and Ala-substituted analogs (compounds **4**−**20**), revealed that the C-terminal hexapeptide of ODN/OP is the minimum sequence retaining full stimulatory activity. Indeed, deletion of any of the amino acid residues of this hexapeptide totally suppressed the biological activity. Similarly, replacement of each individual residue of compound **1** by an Ala moiety

Table 2. Effects of the Octapeptide OP (ODN_{11-18}) and a Series of OP Analogs (10^{-6} M Each, except $Cyclo_{1-8}$[DLeu^5]OP, 10^{-5} M) on the Conversion of [^{3}H]Pregnenolone into 17-Hydroxypregnenolone (17OH-Δ^5P) and Progesterone (P)

			steroids formed (% of control)[b]	
No	analog abbreviation	amino acid sequence[a]	17OH-Δ^5P	P
	control		100.0 ± 12.7	100.0 ± 14.2
1	OP	RPGLLDLK	410.5 ± 42.7[c]	281.7 ± 19.0[c]
2	$cyclo_{1-8}$OP	cRPGLLDLKc	246.9 ± 9.3[c]	271.9 ± 4.7[c]
3	ODN	QATVGDVNTDRPGLLDLK	465.3 ± 16.4[c]	382.7 ± 22.6[c]
4	des-(Arg^1)-OP	PGLLDLK	400.2 ± 46.8[c]	282.1 ± 19.9[c]
5	des-(Arg^1, Pro^2)-OP	GLLDLK	366.6 ± 24.8[c]	266.6 ± 27.0[c]
6	des-(Arg^1, Pro^2, Gly^3)-OP	LLDLK	103.6 ± 22.6[d]	103.8 ± 11.8[d]
7	des-(Lys^8)-OP	RPGLLDL	131.5 ± 23.2[d]	136.6 ± 9.2[d]
8	des-(Leu^7, Lys^8)-OP	RPGLLD	98.2 ± 9.8[d]	98.6 ± 21.8[d]
9	des-(Asp^6, Leu^7, Lys^8)-OP	RPGLL	97.1 ± 4.7[d]	99.6 ± 16.2[d]
10	des-(Arg^1, Lys^8)-OP	PGLLDL	117.5 ± 21.5[d]	137.0 ± 2[d]
11	des-(Arg^1, Pro^2, Lys^8)-OP	GLLDL	98.9 ± 11.2[d]	116.5 ± 13.8[d]
12	des-(Arg^1, Leu^7, Lys^8)-OP	PGLLD	101.3 ± 2.7[d]	101.4 ± 15.5[d]
13	[Ala^1]OP	**A**PGLLDLK	402.7 ± 33.1[c]	276.5 ± 10.1[c]
14	[Ala^2]OP	R**A**GLLDLK	405.0 ± 40.7[c]	265.2 ± 21.6[c]
15	[Ala^3]OP	RP**A**LLDLK	126.0 ± 25.5[d]	99.0 ± 17.0[d]
16	[Ala^4]OP	RPG**A**LDLK	97.1 ± 17.4[d]	104.7 ± 9.9[d]
17	[Ala^5]OP	RPGL**A**DLK	92.8 ± 16.6[d]	108.5 ± 11.8[d]
18	[Ala^6]OP	RPGLL**A**LK	103.6 ± 19.4[d]	100.4 ± 2.4[d]
19	[Ala^7]OP	RPGLLD**A**K	99.5 ± 19.9[d]	103.2 ± 11.6[d]
20	[Ala^8]OP	RPGLLDL**A**	126.0 ± 7.6[d]	137.0 ± 22.8[d]
21	$cyclo_{1-8}$[DLeu^5]OP	cRPGLDLDLKc	99.9 ± 10.6[d]	99.7 ± 11.9[d]

[a] Amino acid sequence reported in the one letter notation. [b] The values were obtained from experiments similar to those presented in Figure 2. Results are expressed as percentages of the amount of each steroid formed in the absence of peptide (mean ± SEM from three independent experiments). [c] $p < 0.001$, by one-way ANOVA followed by a post hoc Bonferroni's test. [d] Not statistically different.

(compounds **15**−**20**) abrogated the neurosteroidogenic activity of the peptide. Interestingly, the primary structure of this hexapeptide has been remarkably well preserved from amphibians to mammals, with only one conservative substitution (Met Leu at position 2) between the frog sequence[37] and the rat/mouse sequence,[40,41] indicating that strong evolutionary pressure has acted to conserve the structure of this peptide. Consistent with these data, it has been previously reported that the C-terminal ODN hexapeptide mimics both the ability of ODN to displace [^{3}H]β-carboline binding from brain membrane receptors and the proconflict activity of ODN, albeit the activity of the former is 4 times lower than that of the latter.[3]

It is now clearly established that DBI and its processing products can interact with either CBR associated with the $GABA_A$ receptor complex[3,42] or PBR located on the outer mitochondrial membrane[20,21] and at the plasma membrane level.[22,33] In addition, it has been shown that endozepines also activate a metabotropic membrane receptor positively coupled to phospholipase C through a PTX-sensitive G-protein.[9,10] To determine the type of receptors mediating the action of compounds **1** and **2** on neurosteroidogenesis, we have investigated the effect of selective antagonists on OP- and $cyclo_{1-8}$-OP-evoked neurosteroid biosynthesis. The specific CBR antagonist flumazenil abolished the effect of compound **1** and significantly decreased the effect of compound **2** on the conversion of [^{3}H]Δ^5P into radioactive steroids, including 17OH-Δ^5P, DHEA, 17OH-P, and P. Conversely, compound **21**, which acts as a specific antagonist of the metabotropic ODN receptor,[36] did not affect basal neurosteroid biosynthesis and did not impair the stimulatory effect of compounds **1** and **2** on neurosteroid formation. These observations indicate that the actions of compounds **1** and **2** on the synthesis of Δ^4-3-keto-neurosteroids and Δ^5-3β-hydroxy-neurosteroids are mediated through activation of CBR. In agreement with this notion, we have previously demonstrated the presence of $GABA_A$/CBR receptor subunits on 3β-HSD-containing neurons.[43]

Conclusion

In summary, the present study has shown that the stimulatory activity of ODN on neurosteroid biosynthesis resides in the C-terminal domain of the peptide and that the minimum sequence possessing full biological activity is the hexapeptide GLLDLK. Because neurosteroids can mimic the behavioral and neurochemical effects of endozepines on food intake,[44] anxiety,[1,45,46] convulsions,[47] and nerve regeneration,[48] this structure−activity relationship study may open new vistas for the design of innovative therapeutic agents.

Experimental Section

Peptide Synthesis. ODN (QATVGDVNTDRPGLLDLK), OP (RPGLLDLK), $cyclo_{1-8}$OP, OP fragments, L-alanine analogs of OP, and $cyclo_{1-8}$[DLeu^5]OP (see Table 1) were synthesized (0.25 mmol scale for ODN, $cyclo_{1-8}$OP, and $cyclo_{1-8}$[DLeu^5]OP; 0.1 mmol scale for all other peptides) on a Fmoc-Lys(Boc)-HMP resin, a Fmoc-Ala-HMP resin, or a Fmoc-Asp(PEG-PS)-OAl resin using an Applied Biosystems model 433A or a Pioneer PerSeptive Biosystems peptide synthesizer and the standard procedures as previously described.[24,36] All Fmoc-amino acids (1 mmol, 4 equiv or 10 equiv) were coupled by in situ activation with HBTU/HOBt (1.25 mmol/1.25 mmol, 5 equiv or 12.5 equiv) and DIEA (2.5 mmol, 10 equiv or 25 equiv) in NMP or DMF. Reactive side chains were protected as follows: Gln and Asn, trityl (Trt) amide; Thr, *tert*-butyl (*t*Bu) ether; Asp, *O-tert*-butyl (O*t*Bu) ester; Arg, pentamethyldihydrobenzofuran (Pbf) sulfonylamide and Lys, *t*-butyloxycarbonyl (Boc) carbamate. For the cyclic peptides $cyclo_{1-8}$OP and $cyclo_{1-8}$[DLeu^5]OP, deprotection of the allyl ester was performed manually by Pd(0) under Ar and on-resin head-to-tail cyclization was performed twice by addition of HBTU/DIEA (0.8 mmol, 8 equiv, 1:1 mol/mol), as previously described.[36] Peptides were deprotected and cleaved from the resin as previously described.[24] All peptides were purified by RP-HPLC, and the purified peptides were characterized by MALDI-TOF mass spectrometry.

The peptides were analyzed by RP-HPLC on a 0.46 × 25 cm Vydac 218TPS4 C_{18} column and on a 0.46 × 25 cm Vydac 219TPS4 diphenyl column (Alltech, Templemars, France) equili-

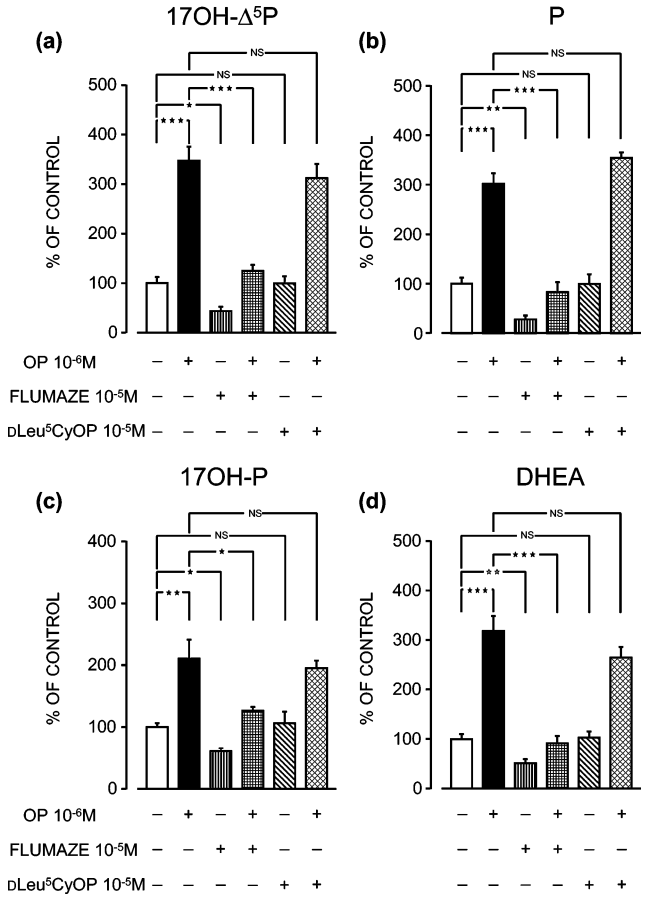

Figure 4. Effects of OP (compound **1**; 10^{-6} M) in the absence or presence of the central-type benzodiazepine receptor antagonist flumazenil (10^{-5} M) or the metabotropic endozepine receptor antagonist cyclo$_{1-8}$[DLeu^5]OP (compound **21**; 10^{-5} M) on the conversion of [^{3}H]-pregnenolone into (a) 17-hydroxypregnenolone (17OH-Δ^5P), (b) progesterone (P), (c) 17-hydroxyprogesterone (17OH-P), and (d) dehydroepiandrosterone (DHEA) by frog hypothalamic explants. The values were obtained from experiments similar to those presented in Figure 2. Results are expressed as percentages of the amount of each steroid formed in the absence of drugs. Each value is the mean (±SEM) of four independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by one-way ANOVA followed by a post hoc Bonferroni's test; NS, not statistically different.

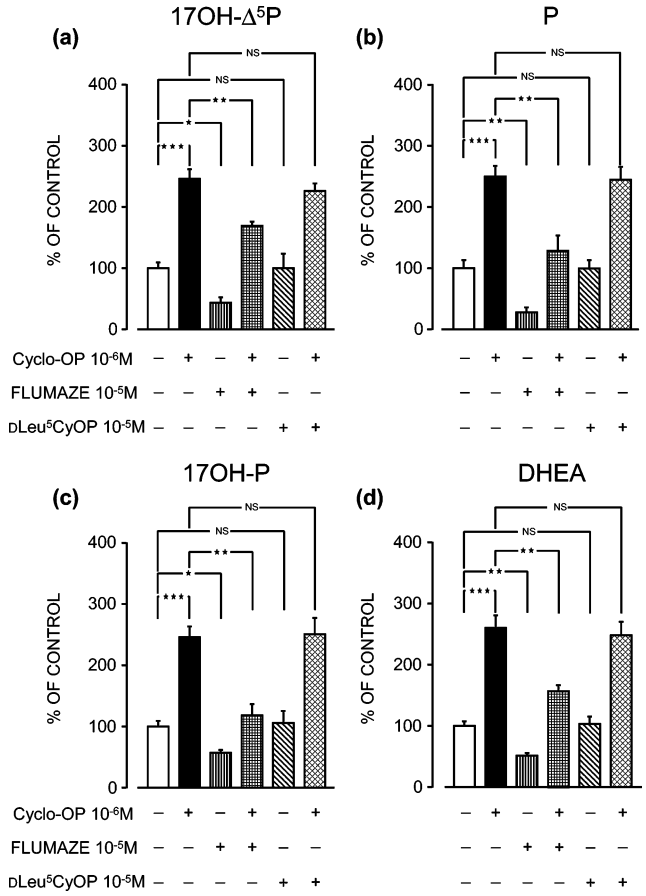

Figure 5. Effects of cyclo$_{1-8}$OP (compound **2**; 10^{-6} M) in the absence or presence of the central-type benzodiazepine receptor antagonist flumazenil (10^{-5} M) or the metabotropic endozepine receptor antagonist cyclo$_{1-8}$[DLeu^5]OP (compound **21**; 10^{-5} M) on the conversion of [^{3}H]-pregnenolone into (a) 17-hydroxypregnenolone (17OH-Δ^5P), (b) progesterone (P), (c) 17-hydroxyprogesterone (17OH-P), and (d) dehydroepiandrosterone (DHEA) by frog hypothalamic explants. The values were obtained from experiments similar to those presented in Figure 2. Results are expressed as percentages of the amount of each steroid formed in the absence of drugs. Each value is the mean (±SEM) of four independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by one-way ANOVA followed by a post hoc Bonferroni's test; NS, not statistically different.

brated with a solution of acetonitrile/water/TFA (10:89.9:0.1, by volume, and 2:97.9:0.1, by volume, respectively) at a flow rate of 1 mL/min during 5 min. The concentration of acetonitrile on the eluting solvent was raised to 60% over 25 min for the C_{18} column and to 34% over 16 min for the diphenyl column.

Chemicals and Reagents. All L-amino acid residues, the preloaded resins, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxy-benzotriazole (HOBt), dimethylformamide (DMF), and *N*-methylpyrrolidin-2-one (NMP) were obtained from Applera-France (Courtaboeuf, France). [^{3}H]Δ^5P (specific activity, 21 Ci/mmol) was purchased from New England Nuclear Life Science Products (Paris, France). Cortisol (F), DHEA, diisopropylethylamine (DIEA), P, T, 11-deoxycortisol (S), 17OH-P, 17OH-Δ^5P, *N*-2-hydroxy-ethyl-piperazine-*N*′-2-ethane sulfonic acid (HEPES), propylene glycol, tetrakis(triphenylphosphine)-palladium(0) ([Ph$_3$P]$_4$Pd), trifluoroacetic acid (TFA), and other reagents were from Sigma-Aldrich (St. Louis, MO). Methanol, acetonitrile, and dichloromethane were from Carlo Erba (Val-de-Reuil, France). Bovine serum albumin (BSA) was from Boerhinger (Paris, France). Flumazenil (Ro15−1788) was a generous gift from Dr. Heafly (Hoffmann-La Roche, Basel, Zwitzerland).

Animals. Adult male frogs (*Rana esculenta*; body weight ranging from 30 to 40 g) were obtained from a commercial source (Couétard, Saint-Hilaire de Riez, France). The animals were maintained under a 12 h light−12 h dark schedule (lights on from 06:00−18:00 h) in a temperature-controlled room (8 ± 0.5 °C). Frogs were kept under running water for at least one week before being sacrificed. To limit possible variations of neurosteroid biosynthesis due to circadian rhythms,[49] all animals were killed between 09:30 and 10:30 a.m. Animal manipulations were performed according to the recommendations of the French Ethical Commitee and under the supervision of authorized investigators.

Measurement of 3β-HSD and P450$_{C17}$ Activities. The experimental procedure applied to study the conversion of [^{3}H]Δ^5P into different metabolites has been previously described.[32,35,50] Briefly, for each experimental value, the hypothalami from four frogs (approximately 10 mg of tissue) were rapidly dissected out and each hypothalamus was cut into two halves. The tissue fragments were preincubated for 15 min in 1 mL of Ringer's solution consisting of 15 mM HEPES buffer, 112 mM NaCl, 15 mM $NaHCO_3$, 2 mM $CaCl_2$, and 2 mM KCl, supplemented with 2 mg glucose/mL and 0.3 mg BSA/mL. The incubation medium was gassed with a 95% O_2/5% CO_2 mixture and the pH was adjusted to 7.4. The hypothalamic explants were incubated at 24 °C for 2 h in 500 μL Ringer's medium containing 10^{-6} M [^{3}H]Δ^5P and 4% propylene glycol, in the absence or presence of test substances. At the end of the incubation period, the tissues were rinsed four times with ice-cold Ringer's buffer, and the reaction was stopped by

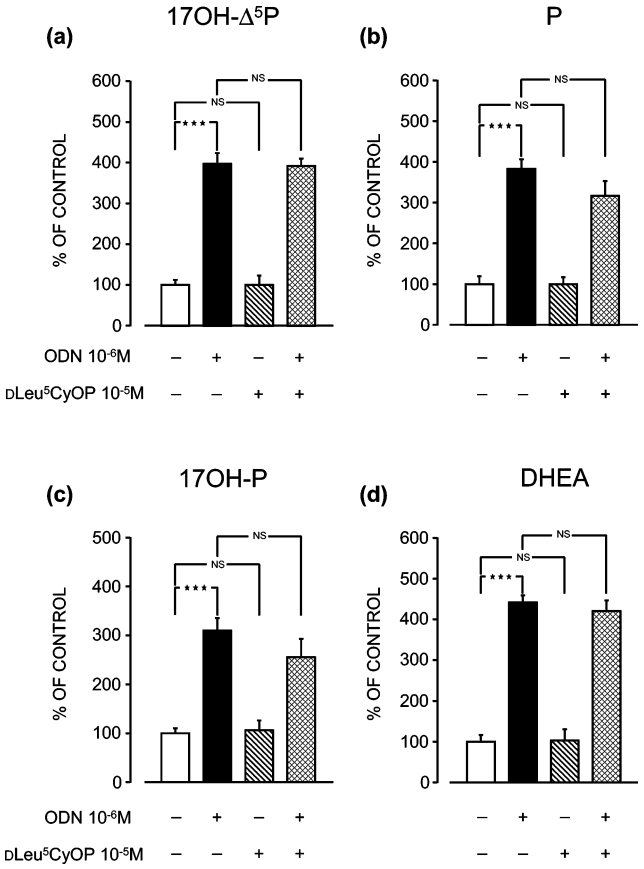

Figure 6. Effects of ODN (compound **3**; 10^{-6} M) in the absence or presence of the metabotropic endozepine receptor antagonist cyclo$_{1-8}$[DLeu^5]OP (compound **21**; 10^{-5} M) on the conversion of [^{3}H]-pregnenolone into (a) 17-hydroxypregnenolone (17OH-Δ^5P), (b) progesterone (P), (c) 17-hydroxyprogesterone (17OH-P), and (d) dehydroepiandrosterone (DHEA) by frog hypothalamic explants. The values were obtained from experiments similar to those presented in Figure 2. Results are expressed as percentages of the amount of each steroid formed in the absence of drugs. Each value is the mean (±SEM) of four independent experiments. ***$p < 0.001$ by one-way ANOVA followed by a post hoc Bonferroni's test; NS, not statistically different.

adding 1 mL of trichloroacetic acid. The tissues were homogenized using a glass potter homogenizer, and the steroids were extracted three times with 1 mL of dichloromethane. The organic phase containing the steroids was evaporated under nitrogen, and the tissue extracts were dissolved in a solution consisting of 65% water/TFA (99.9:0.1; vol/vol; soln A) and 35% methanol/water/TFA (90:9.98:0.02; vol/vol/vol; soln B) and prepurified on Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) equilibrated with a solution made of 65% soln A and 35% soln B. Steroids were eluted with 4 mL of a solution made of 10% soln A and 90% soln B. The solvent was evaporated in a Speed-Vac concentrator (Savant, Hicksville, NY), and the extracts were kept dry at 4 °C until RP-HPLC analysis.

High Performance Liquid Chromatography. Sep-Pak-prepurified extracts were analyzed by RP-HPLC as previously described[35] using a Gilson model 305 master pump acting as a system controller, a Gilson model 306 slave pump controlled by the previous pump, a Gilson model 115 variable wavelength UV detector set at 240 nm (Gilson S.A., Villier-le-Bel, France) and a Rheodyne model 7125 injector (Rheodyne Inc, California). A 0.39 × 30 cm Nova-Pak C_{18} column (Waters Associates) equilibrated with 60% soln A and 40% soln B was used for analysis. Each dry extract was dissolved in 400 μL of a solution consisting of 60% soln A and 40% soln B, and the whole sample was injected at a flow rate of 1 mL/min. The steroids formed from [^{3}H]Δ^5P were separated using a gradient of soln B (40−100% over 104 min) including four isocratic steps at 40% (0−10 min), 64% (39−59 min), 80% (69−79 min), and 100% soln B (94−104 min). Tritiated compounds eluted from the HPLC column were detected by using a flow scintillation analyzer (Radiomatic Flo-OneB A-500, Packard, Meridien, CT) equipped with a computer that automatically integrates the radioactivity contained in each peak.

Synthetic steroids used as reference standard were chromatographed under the same conditions as the tissue extracts and detected by UV absorption. To determine the precision of the measurement of the radioactivity contained in each peak, a mixture of tritiated steroids ([^{3}H]P, [^{3}H]17OH-P, [^{3}H]T, and [^{3}H]Δ^5P) was analyzed by HPLC, on different days, using the same conditions as for the hypothalamic samples. The between-day coefficients of variation were 6.6% for [^{3}H]P, 5.1% for [^{3}H]17OH-P, 5.4% for [^{3}H]T, and 3.2% for [^{3}H]Δ^5P.

Quantification of Steroid Biosynthesis and Statistical Analysis. The amounts of radioactive steroids formed by conversion of [^{3}H]Δ^5P were expressed as a percentage of the total radioactivity contained in all peaks resolved by RP-HPLC, including [^{3}H]Δ^5P itself. Each value is the mean of four independent experiments from distinct hypothalamic extracts. Statistical analysis was performed by ANOVA followed by a Bonferroni's multiple comparison test.

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