Synthesis and Biological Activity of *O*-Glycosylated Morphiceptin Analogues†‡

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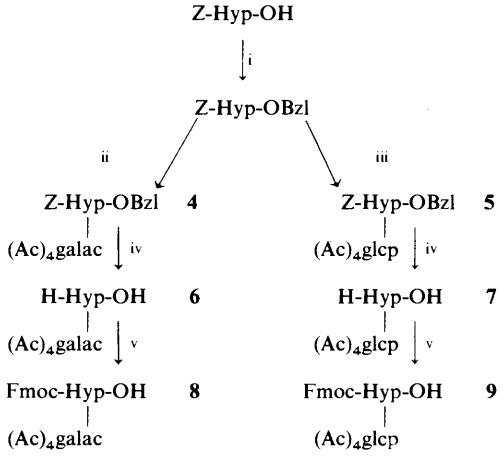
The synthesis and biological activity of [Hyp^4]morphiceptin and two glycosyl derivatives are reported. Glycopeptide amides were obtained using Fmoc solid-phase chemistry and mild conditions for cleavage from a tris(alkoxy)benzylamide (PAL) resin. Analogues were evaluated in the guinea pig ileum *in vitro* assay and in *in vivo* tail-flick and paw-pressure antinociceptive tests after intrathecal administration in rats. Substitution of Pro^4 by Hyp^4, and further derivatization of Hyp with glucose or galactose, resulted in an unexpected decrease in biological activity with respect to morphiceptin.

Morphiceptin (Tyr-Pro-Phe-Pro-NH_2), or β-4-casomorphin-amide, is a tetrapeptide amide with high opioid agonist activity.[2] The peptide is derived from bovine β-casein, a natural source of exorphins.[3] With the goal of improving potency and/or selectivity towards the μ-opioid receptor, a considerable number of morphiceptin derivatives have been evaluated in the last few years.[4–7] Substitution of Pro^4 by D-Pro or D-Val, and *N*-methylation of Phe^3, proved to be effective modifications[8] to obtain active μ-selective analogues. Another family of morphiceptin-like structures are derived from human β-casein, and contain Val^4 in place of Pro^4. The human peptide with a *C*-terminal amide, valmuceptin, is more potent than morphiceptin and has similar selectivity towards the μ-opioid receptor.[9,10]

The present work considers the effects of replacement of Pro^4 in morphiceptin by Hyp^4, as well as analogues where the side-chain of Hyp was further modified by glycosylation with a glucopyranosyl or galactopyranosyl moiety. We showed earlier that the introduction of the same carbohydrate side-chains onto [D-Met^2,Hyp^5] enkephalinamide resulted in a significant increase of antinociceptive activity.[11,12] Similar but less potent effects of glycosylation in other opioid peptides have been reported elsewhere.[13]

Scheme 1 Synthesis of glycosylated building blocks from 4-hydroxyproline



Reagents: i, Cs_2CO_3, BzlCl; ii, BF_3·Et_2O, $(Ac)_5$galac; iii, BF_3·Et_2O, $(Ac)_5$glcp; iv, H_2/Pd–C; v, Fmoc-OSu

Results and Discussion

The synthesis of [Hyp^4]morphiceptin **1** and its galactopyranosyl and glucopyranosyl derivatives **2** and **3** has been carried out under mild conditions as outlined previously.[1] Our SPGPS procedure entails incorporation of glycosylated building blocks **8** or **9** (Scheme 1)¶ onto a tris(alkoxy)benzylamide (PAL) polystyrene resin,[15,16] and subsequent stepwise coupling of the remaining Fmoc-amino acids (Scheme 2). Acetyl protecting groups on the sugar moieties were removed by hydrazinolysis *while the peptide remained anchored to the resin*, and final quantitative cleavage from the resin, as well as deblocking of the *tert*-butyl ether on tyrosine, was achieved with TFA–CH_2Cl_2 (7:3). The desired *C*-terminal glycopeptide amides were obtained in reasonable initial purities, and were homogeneous after reversed-phase semi-preparative HPLC. The final purities (>99%) were established by analytical HPLC (Fig. 1) and further support for the structures was derived by FAB-MS. Results by our methodology compare favourably with those for related purposes reported in the recent literature.[17–23]

The synthetic morphiceptin derivatives, as well as morphiceptin itself, were examined for their ability to inhibit the electrically induced contractions of guinea pig ileum (GPI)-myenteric plexus preparations.[24] The potencies of the different compounds are expressed as IC_{50} values, and all the values represent the mean of six determinations of cumulative dose–response curves (Table 1). Note that the substitution of Hyp^4

† For a preliminary communication of a portion (chemical synthesis) of this work, see ref. 1. This paper is taken in part from the Ph.D. thesis of E. B., University of Barcelona, November 1989.

‡ Unless specified otherwise, DCC: *N*,*N*′-dicyclohexylcarbodiimide; DIEA: diisopropylethylamine; DMF; *N*,*N*-dimethylformamide; Fmoc: fluoren-9-ylmethoxycarbonyl; galac: galactopyranose; glcp: glucopyranose; HOBt: 1-hydroxybenzotriazole; PAL: 5-[4-(Fmoc-aminomethyl)-3,5-dimethoxyphenoxy] valeric acid; SPGPS: solid-phase glycopeptide synthesis; TFA: trifluoracetic acid; OSu: *N*-hydroxysuccinamide.

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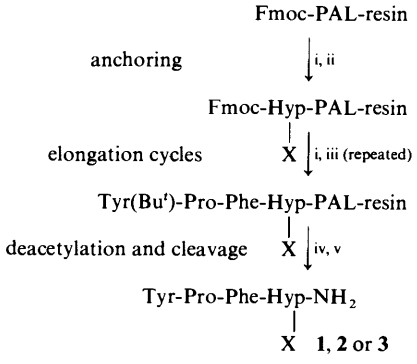
¶ For a preliminary report of an improved route to the required building blocks (demonstrated on Ser and Thr, not on Hyp), see ref. 14.

Scheme 2 Solid-phase glycopeptide synthesis (SPGPS) of [Hyp^4]-morphiceptin **1** and its galactopyranosyl **2** and glucopyranosyl **3** derivatives



Reagents: i, piperidine–DMF (1:4); ii, Fmoc-Hyp(X)-OH and DCC–HOBt, where X = Bu^t for **1**, Galac(Ac)$_4$ (**8** in Scheme 1) for **2**, or Glcp(Ac)$_4$ (**9** in Scheme 1) for **3**; ii, Fmoc-Phe-OH, Fmoc-Pro-OH, or Fmoc-Tyr(Bu^t)-OH with DCC–HOBt; iv, $N_2H_4 \cdot H_2O$, methanol (4:1) (just for deacetylated glycopeptides); v, TFA–CH_2Cl_2 (7:3). Final products: X = H for **1**; X = Galac for **2**; and X = Glcp for **3**

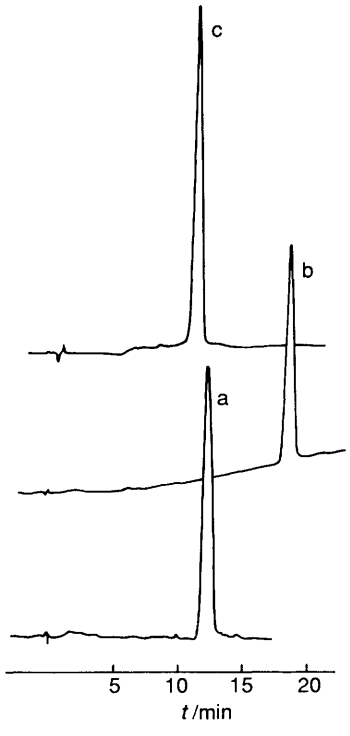


Fig. 1 Elution profiles of purified peptides and glycopeptides. Analytical reversed-phase HPLC column (C_{18}; 1 × 25 cm; 10 μm), with detection at 215 nm and elution with 0.1% aq. TFA–0.1% TFA in MeCN, linear gradient from 10 to 100% organic solvent, at a flow rate of 1 cm^3 min^{-1} for 30 min. (a) [Hyp^4]morphiceptin; (b) $O^{1,4}$-(tetra-*O*-acetyl-β-D-glucopyranosyl)[Hyp^4]morphiceptin; (c) $O^{1,4}$-(β-D-glucopyranosyl)[Hyp^4]morphiceptin. The epimeric glycopeptides (galactose replaces glucose) were indistinguishable by HPLC.

for Pro^4 in morphiceptin provided an analogue which was six-fold less effective in the GPI bioassay. Glycosylation of [Hyp^4]morphiceptin **1** with a glucopyranose residue had no further effect on the GPI activity of the resultant analogue **3**, but the corresponding galactopyranose analogue **2** had substantially reduced activity; with its IC_{50}-value for the GPI bioassay being in the 10 μmol dm^{-3} range the Gal analogue was five times less effective than [Hyp^4]morphiceptin **1** and 25 times less effective than morphiceptin.

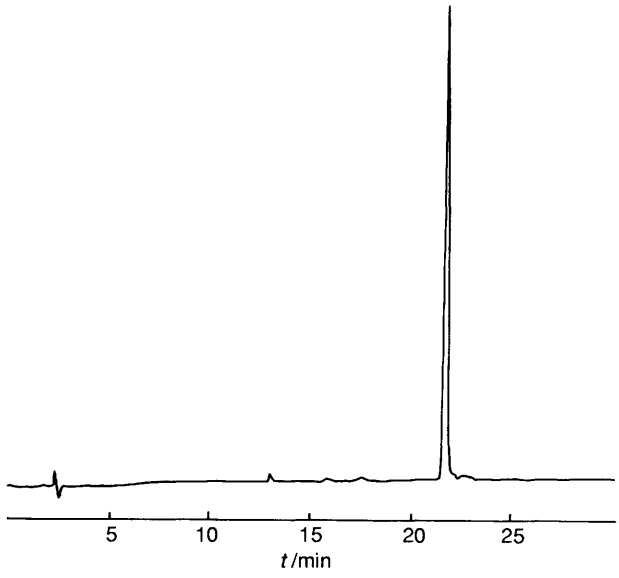


Fig. 2 Elution profile of purified $O^{1,4}$-(tetra-*O*-acetyl-β-D-glucopyranosyl)-Fmoc-Hyp-OH. Analytical reversed-phase column (C_{18}; 1 × 25 cm; 10 μm), with detection at 215 nm and elution with 0.1% aq. TFA–0.1% TFA in MeCN, linear gradient from 10 to 100% organic solvent, at a flow rate of 1 cm^3 min^{-1} for 30 min. The epimeric derivative (galactose replaces glucose) was indistinguishable by HPLC.

Table 1 Agonist activity of synthetic morphiceptin derivatives in the GPI bioassay

Compound	IC_{50}[a] ± s.e.m.[b] in GPI (μmol dm^{-3})
Morphiceptin	0.392 (0.36–0.42)
[Hyp^4]Morphiceptin **1**	2.54 (2.24–2.89)
($O^{1,4}$-β-D-glcp)[Hyp^4]Morphiceptin **3**	2.12 (1.93–2.32)
($O^{1,4}$-β-D-galac)[Hyp^4]Morphiceptin **2**	9.87 (8.96–10.86)
Morphine	0.085 (0.073–0.099)
DAMGO[c]	0.009 (0.007–0.011)

[a] IC_{50}-values were calculated from the cumulative dose–response curves and are expressed as the mean of six experiments. [b] s.e.m. = standard error of the mean. [c] DAMGO = Tyr-D-Ala-Gly-MePhe-Glyol, μ-selective agonist.[25]

Table 2 Antinociceptive activity of the synthetic morphiceptin analogues evaluated in the tail-immersion and paw-pressure tests

Compound		Time[a] (*t*/min)	Tail immersion[b]	Paw pressure[b]
Morphine	10 μg	10	70–80	70–80
[Hyp^4]Morphiceptin **1**				
	10 μg	10	10–15	15–20
	20 μg	15	50–60	50–65
($O^{1,4}$-β-D-glcp)[Hyp^4]-Morphiceptin **3**	10 μg	10	5–10	5–10
	20 μg	15	30–40	30–40
($O^{1,4}$-β-D-galac)[Hyp^4]-Morphiceptin **2**	10 μg	10	*c*	*c*

[a] Times corresponding to the maximum analgesic effect observed during each experiment. [b] Data represent percent of analgesia. Control values are equivalent to 0% analgesia [c] No antinociceptive activity was observed when testing the galactosyl derivative.

For *in vivo* tests (Table 2), peptides and glycopeptides (and morphine as a reference compound) were injected intrathecally into chronically catheterized rats[26] at doses of 10 and 20 μg, and their antinociceptive activity was studied by the tail-flick and paw-pressure tests at intervals of 5 min for the first 30 min, and subsequently every 15 min until a total time of 90 min

had elapsed. By these experiments, it was shown that [Hyp^4]-morphiceptin had somewhat less antinociceptive activity with respect to morphine, and activities of the glycosylated derivatives were even further suppressed. Galactosyl derivative **2** had the least effect, in parallel with the *in vitro* observations.

The present results dealing with effects of glycosylation on the biological activities of morphiceptin are in contradistinction to our earlier findings of enhanced antinociceptive activity for glycosylated enkephalinamides.[11,12] Our findings add to the evidence[2-7] of significant differences between the biological activities of enkephalins and β-casomorphins. Of additional interest for both families of opioid peptides are the changes in biological activity corresponding to the change of the epimeric sugars galactose and glucose. It seems plausible that the sugar moiety influences the active conformation of the peptide portion.[28] The preparation and biological testing of further new analogues will be required in order to clarify structure–activity relationships for opioid glycopeptides.

Experimental

Fmoc and other amino acid derivatives were obtained from Bachem (Basel, Switzerland). The side-chains of tyrosine and 4-hydroxyproline were protected as *tert*-butyl ethers. Per-*O*-acetylated sugars were obtained from Aldrich-Chemie (Steinheim, Germany). ^{1}H and ^{13}C NMR spectra were observed with a Bruker (80 MHz) or a Varian XL200 (200 MHz) spectrometer for solutions in $CDCl_3$. Chemical shifts are reported as δ-values downfeld from internal tetramethylsilane as reference, and *J*-values are given in Hz. Fast-atom bombardment mass spectra (FAB-MS) were determined by the Laboratory of Mass Spectrometry, CID-CSIC, Barcelona, using an MS9-VG updated system equipped with a VG-11-250 data system, and with glycerol or thioglycerol as solvent. TLC was performed on silica gel plates (0.25 mm) from Merck, with the following solvent systems: diisopropyl ether–HOAc (19:1) R_{FA}, EtOAc–HOAc (19:1) R_{FB} and $CHCl_3$–MeOH–HOAc (95:5:3) R_{FC}; with detection by ninhydrin (for amines), and chlorine–tolydine or orcinol–$FeCl_3$ (for sugars). All solvents for solid-phase synthesis were analytical grade. DMF was dried and stored over 4-Å molecular sieves; immediately before use, the DMF was also freed from volatile amines by a helium stream and was then passed through a 0.25 μm filter. Solid-phase couplings were monitored by the Kaiser ninhydrin test.[29] Unless specified otherwise, reactions were conducted at ambient temperature with no precautions to exclude atmospheric oxygen.

5-[4-(*Fmoc-aminomethyl*)-3,5-*dimethoxyphenoxy*]*valeryl-amidomethylpolystyrene Resin* (*PAL-Resin*).—An aminomethyl–polystyrene resin (0.15 g, 0.65 mequiv g^{-1}, 0.1 mmol, Novabiochem) was swollen with CH_2Cl_2 (3 × 1 min) and washed successively with DIEA–CH_2Cl_2 (1:19) and CH_2Cl_2. The PAL linker[15,16] (101 mg, 0.2 mmol) and HOBt (30 mg, 0.2 mmol) were dissolved in DMF (0.75 cm^3) and a solution of DCC (42 mg, 0.2 mmol) in the minimum of CH_2Cl_2 (0.2 cm^3) was added. After 15 min, the formed dicyclohexylurea was filtered off, the CH_2Cl_2 was evaporated off and the resulting DMF solution was added to the swollen resin. After 2 h, a ninhydrin test[29] was negative. The resultant PAL-resin was washed successively with DMF, CH_2Cl_2, and methanol, dried *in vacuo*, and stored.

H-Tyr-Pro-Phe-Hyp-NH$_2$ **1**.—The PAL-resin (0.16 g, 0.1 mmol) was treated with piperidine–DMF (1:4) (10 min) to remove the handle Fmoc group. The appropriate protected amino acids were incorporated in turn by DCC–HOBt-mediated couplings carried out as follows: the Fmoc-amino acid plus HOBt (0.25 mmol each) were dissolved in CH_2Cl_2 (0.75 cm^3) with just enough DMF to assist HOBt solubilization, and this solution was added to the peptide-resin. Next, there was added a soluton of DCC (52 mg, 0.25 mmol) in CH_2Cl_2 (0.5 cm^3) to initiate coupling (which was allowed to proceed for 1 h). Ninhydrin tests[29] indicated that coupling was complete, so the peptide-resin was then washed successively with DMF (5 × 1 min) and CH_2Cl_2 (5 × 1 min), treated with piperidine–DMF (1:4) (1 + 10 min) to remove the Fmoc group, and washed again with DMF (5 × 1 min) and then with CH_2Cl_2 (5 × 1 min). Upon completion of chain assembly, the peptide-resin was cleaved for 2.5 h with TFA–CH_2Cl_2 (7:3) (10 cm^3). The filtrate was collected and combined with further resin washes with TFA–CH_2Cl_2 (7:3) (10 cm^3) and CH_2Cl_2 (5 cm^3). Solvents were removed by evaporation, and the resultant oil was dissolved in water (10 cm^3) and washed with CH_2Cl_2 (3 × 10 cm^3). The aq. layer was then lyophilized, the residue was dissolved in the minimum amount of HOAc, and this solution was lyophilized again. Yield 49 mg (90%); *m/z* (FAB-MS) 538 (M + 1); HPLC (column and conditions as in Fig. 1) t_R 12.9 min, k = 3.9, >99% pure.

Glycosylmorphiceptins **2** *and* **3**.—The procedure used was identical with that already described for the preparation of compound **1**, with the exception that building block **8** or **9** was incorporated in place of Fmoc-Hyp(Bu^t)-OH. As a check for the effectiveness of the synthesis, the acetylated glycopeptide amides were obtained by the same acid-cleavage procedure already given. HPLC (column and conditions as in Fig. 1), t_R 18.2 min; k 5.8.

To obtain the free glycopeptide amides, the glycopeptide-resin was first treated with hydrazine hydrate–methanol (4:1) (1 min + 2 h), and was then washed successively with methanol (5 × 1 min) and CH_2Cl_2 (3 × 1 min), and this was followed by the usual acid cleavage from the resin. For both products **2** and **3**: *m/z* (FAB-MS) 700 (M + 1); HPLC (column and conditions as in Fig. 1), t_R 11.6 min; k 3.5.

The final glycosylmorphiceptin analogues were purified by semipreparative HPLC on a 1 × 25 cm column (C_{18}; 10 μm) with detection at 215 nm. Elution was at a flow rate of 4 cm^3 min^{-1} under isocratic conditions with 0.1% aq. TFA–0.1% TFA in MeCN (3:1) or, alternatively, a linear gradient was run at a flow rate of 2.5 cm^3 min^{-1} from 10 to 100% organic solvent during 30 min.

Z-Hyp-OBzl.—According to the general esterification method of Wang *et al.*,[30] Z-Hyp-OH (2.0 g, 7.5 mmol) was treated with Cs_2CO_3 (1.22 g, 3.75 mmol) and benzyl chloride (1.73 cm^3, 15.03 mmol) to give the title product (2.4 g, 94%), R_{FC} 0.58; δ_H(80 MHz) 1.9–2.3 (2 H, m, β-Hyp and OH), 3.6 (2 H, d, δ-Hyp), 4.6 (2 H, m, α- and γ-Hyp), 5.0–5.2 (4 H, ds, PhCH_2) and 7.2–7.4 (10 H, m, Ph).

N$^{\alpha}$-*Z*-O^2-(*Tetra*-O-*acetyl*-β-D-*galactopyranosyl*/*glucopyranosyl*)-*Hyp-OBzl* **4** *and* **5**.—BF_3·Et_2O (0.89 cm^3, 7.0 mmol) was added dropwise at 0 °C to a solution of Z-Hyp-OBzl (0.5 g, 1.4 mmol) and penta-*O*-acetyl-β-D-galactopyranose (0.55 g, 1.4 mmol) in CH_2Cl_2 (50 cm^3). The reaction mixture was maintained for 6 h at 10 °C, and then was quenched in ice (200 g). The aq. layer was washed with CH_2Cl_2 (3 × 25 cm^3), and the combined organic extracts were washed successively with 20% aq. $NaHCO_3$ (3 × 40 cm^3) and water (3 × 40 cm^3), dried (Na_2SO_4), and concentrated. The resultant oil was purified by flash chromatography on silica gel (40–63 μm) in a 5 × 15 cm column eluted consecutively with diisopropyl ether–HOAc (19:1) and EtOAc at a flow rate of 5 cm^3 min^{-1} to give compound **4** (0.48 g, 50%). The glucosyl derivative **5** (0.29 g, 30%) was prepared following the same procedure with penta-acetylglucopyranose, R_{FA} 0.13; *m/z* (FAB-MS) (thioglycerol) 686 (M + 1) for both products **4** and **5**.

N^{α}-*Fmoc*-O^{1}-(*tetra*-O-*acetyl*-β-D-*galactopyranosyl*/*gluco-pyranosyl*)-*Hyp-OH* **8** *and* **9**.—Intermediates **4** and **5** (514 mg, 0.75 mmol) were separately dissolved in EtOAc (50 cm^3), and then water (5 cm^3) and 10% palladium–charcoal (50 mg) were added to each solution. These suspensions were hydrogenolysed overnight at atmospheric pressure to cleave the Z and Bzl groups. The resulting tetra-*O*-acetylglycosylated hydroxyprolines **6** and **7** were separately dissolved in water (10 cm^3) and treated with Fmoc-OSu[31] (243 mg, 0.72 mmol) in MeCN (5 cm^3) by dropwise addition of triethylamine to achieve a steady pH of 8.5. After 1 h, the reaction mixtures were concentrated under reduced pressure and lyophilized. The resultant oils were suspended in EtOAc and purified on silica gel (40–63 μl) eluted with hexane–EtOAc–HOAc (2:17:1). Compound **8** (341 mg, overall 30% from 1-Hyp-PBzl) had R_{FB} 0.4; *m/z* (FAB-MS) 683 (M + 1); HPLC (column and conditions as in Fig. 2), t_R 22.4 min, *k*11.0; δ_H(200 MHz) 1.99–2.15 (12 H, 4 s, Ac), 2.15 (2 H, m, β-Hyp), 3.78 (2 H, m, δ-Hyp), 4.15 (1 H, d, α-Hyp), 4.30 (1 H, m, γ-Hyp), 4.58 (1 H, d, $J_{1,2}$ 7.5, 1-H galac β-anomer), 5.05 (1 H, dd, 3-H galac), 5.17 (1 H, dd, 2-H galac), 5.39 (1 H, d, 4-H galac) and 7.30–7.82 (8 H, m, arom Fmoc); δ_C(50 MHz) 99.7 and 99.3 (C-1 galac β-anomer).

Compound **9** (328 mg, overall 20% from Z-Hyp-OBzl) had the same R_{FB}- and *k*-value as isomer **8**; *m/z* (FAB-MS) 683 (M + 1); δ_C(50 MHz) 99.7 and 99.2 (C-1 glcp β-anomer).

Bioassay.—Inhibition of electrically evoked contractions of the longitudinal muscle in the myenteric plexus of guinea pig ileum (GPI) preparation was carried out as reported in the literature.[24] A siliconized organ cell-bath (13 cm^3) and isometric transducer were used; preparations were stimulated at supramaximal voltage at a rate of 0.1 Hz and maintained at 37 °C in Krebs-hydrogen carbonate buffer bubbled with O_2–CO_2 (95:5). Drugs and peptides were dissolved initially in water, and dilutions to the concentrations for testing were made with Krebs solution. Calculations of cumulative dose–response curves were performed with the GRAPHPAD statistical program (from ISI).

Antinociceptive Tests.—Male Sprague-Dawley rats (280–320 g; from Interfauna, Barcelona) were individually housed in a temperature-controlled environment (21 ± 1 °C) on a 12 h light–dark cycle (08.00–20.00 h light) and with free access to food and water. Rats were chronically catheterized intrathecally by the technique of Yaksh and Rudy.[26] The animals were allowed to recover for 6 days after surgery before being tested. They were brought into the testing room 1–2 h before the start of experiments. Each animal was used only once and experiments were performed under double-blind conditions. All injections were made with a fixed-needle Hamilton syringe at the rate of 20 mm^3 min^{-1}; drugs were dissolved in sterile saline, injected in a volume of 10 mm^3, and flushed with sterile saline (5 mm^3). Rats showing control responses higher than 75% of the chosen cut-off values were discarded before further testing. Rats injected just with physiological saline were used in some experiments and no effect was observed in the latencies for either test.

The tail-flick test was performed with a Socrel apparatus, setting a cut-off time of 10 s, and locating a 10-cm distal point of the tail. Mechanical nociceptive thresholds were determined using the paw-pressure test (left hind paw). An analgesymeter (Ugo Basile, Milan) was set up to apply a force increasing from zero to 1000 g at a rate of 80 g s^{-1}. The end-point was noted when the rat vocalized or struggled vigorously. Each animal was studied first in the tail-flick test, followed by the paw-pressure test, from 1 min after injection up to 90 min of experimentation. Only the results from animals with cannulae positioned correctly in the lumbar subarachnoid space (T_{12}–L_3) were included in the data analysis.

Acknowledgements

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