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Synthesis and antinociceptive activity of cyclic endomorphin-2 and morphiceptin analogs

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ABSTRACT

Cyclic analogs of the opioid peptides endomorphin-2 and morphiceptin of the type Tyr-X-Phe-Phe-Y-NH_2 and Tyr-X-Phe-D-Pro-Y-NH_2 (X = Lys or Asp and Y = Lys or Asp), respectively, were synthesized in order to test their structure–activity relationships. Antinociceptive activity of the new analogs was assessed in the hot-plate test after intracerebroventricular administration in mice. The strong analgesic effect was observed for the analogs with Asp in position 2, while the analogs with Lys in the second position were inactive. Antinociception caused by Asp^2 analogs was dose-dependent and reversed by the concomitant administration of the universal opioid antagonist naloxone and by the selective κ antagonist, nor-BNI. However, receptor binding studies revealed poor affinity of all cyclic analogs at the μ-opioid receptor and no affinity at δ- and κ-opioid receptors. It is most likely that the new cyclic analogs produced their antinociception by the release of dynorphin A, which subsequently acted on the κ-opioid receptor.

1. Introduction

The opioids were among the earliest neuropeptides identified in the nervous system [1]. Opioid receptors are most abundant in the central nervous system, but have also been localized in many peripheral tissues of the mammalian organism [2]. It is well established from work carried out in many laboratories over the last 30 years that there are three well-defined types of opioid receptors: the μ-, δ- and κ-opioid receptors [3–5]. Opioid binding sites were found to be involved in pain relief effects, but in a different degree. The μ-receptor mediates the most potent antinociceptive effect, accompanied, however, by the development of tolerance and dependence. The δ- and κ-receptor ligands have lower efficacy, but also a reduced addictive potential [6]. The endogenous ligands for all three receptor types were isolated from the mammalian brain in 1980's, namely δ-selective enkephalins [7] and κ-selective dynorphins [8], while μ-selective endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) were discovered quite recently [9].

Much earlier the number of milk protein fragments was shown to behave as opioid receptor ligands able to address opioidergic systems in mammalian organisms [10,11]. Morphiceptin (Tyr-Pro-Phe-Pro-NH_2), a tetrapeptide amide isolated from the enzymatic digest of bovine casein, was found to bind with fairly high affinity and to be extremely selective for the μ-receptor [12,13].

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The structures of endomorphin-2 and morphiceptin differ only by the amino acid in the fourth position (Phe and Pro, respectively). Both peptides have a unique N-terminal Tyr-Pro sequence, different from the N-terminal tetrapeptide sequence (Tyr-Gly-Gly-Phe), characteristic for typical opioids, such as enkephalins and dynorphins [14].

As part of our efforts to obtain μ-selective opioid peptides with improved analgesic profile, we have synthesized several conformationally restricted analogs of endomorphin-2 and morphiceptin. These analogs were obtained by cyclization through an amide bond between side-chain amino and carboxy groups of diamino and dicarboxy amino acids, introduced into the peptide sequence in positions 2 and 5. The resulting peptides were screened for their binding activity at μ-, δ- and κ-opioid receptors in the rat brain membranes and for their antinociceptive activity in the hot-plate test in mice.

2. Materials and methods

2.1. Peptide synthesis

Peptides were synthesized by standard solid-phase procedure as described before [15], using techniques for Boc(*t*-butyloxycarbonyl)-protected amino acids on *p*-methylbenzhydrylamine (MBHA) resin (100–200 mesh, 0.8 mM/g, Novabiochem, La Jolla, USA). N^{ε}-amino group of Lys was protected by Fmoc (9-fluorenylmethyloxycarbonyl), β-carboxy group of Asp by OFm (fluorenylmethyl ester) and hydroxy group of Tyr by 2-Br-Z (2-bromo-benzyloxycarbonyl group). Fifty percent trifluoroacetic acid (TFA) in dichloromethane was used for deprotection of Boc-groups and 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) was employed to facilitate coupling. Fully assembled Boc-protected peptides were treated with 20% piperidine in DMF to remove base-labile groups (Fmoc and OFm), followed by cyclization (TBTU and *N*-methyl morpholine). Simultaneous deprotection and cleavage from the resin was accomplished by treatment with 90% anhydrous hydrofluoric acid (HF) and 10% anisole scavenger at 0 °C for 1 h. Crude peptides were purified by RP HPLC on a Vydac C_{18} column (1 cm × 25 cm) using the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 20–90% B over 25 min. Calculated values for protonated molecular ions were in agreement with those obtained using FAB mass spectrometry.

2.2. Opioid receptor binding assays

The opioid receptor binding assays were performed as described previously [16,17]. Binding affinities for μ- and δ-receptors were determined by displacing [^{3}H]Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) and [^{3}H]Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 ([^{3}H]DLT) from rat brain membrane binding sites, and for κ-opioid binding sites were measured by displacement of [^{3}H]U-69593 from guinea-pig brain membrane binding sites. Synaptosomal fraction (600 μg of protein), 2 nM radioligand, bovine serum albumin (500 μg), bacitracin (50 μg), bestatin (10 μg) and soybean trypsin inhibitor (20 μg) were incubated in Tris–HCl buffer (50 mM, pH 7.4, total volume 500 μl) for 1 h at 25 °C. Incubations were terminated by rapid filtration through GF/B Whatman glass fiber strips, soaked in 0.5% PEI. The filters were washed twice using ice-cold Tris–HCl buffer. Non-specific binding was determined in the presence of unlabeled DAMGO or DLT. The amount of the radioactivity bound to the filter was determined after overnight extraction with 3 ml of Creasol I (Nacalai Tescue, Japan), using a Beckman 9800 scintillation counter. The IC_{50} values were determined from logarithmic dose-dependent displacement curves and the values of the inhibitory constant K_i of peptides were calculated according to the equation of Cheng and Prusoff [18]. All reactions were carried out in triplicate.

2.3. Antinociception

The procedures used in this study were in accordance with the European Communities Council Directive (86/609/EEC), and approved by the Local Ethical Committee for Animal Research.

Male Swiss albino mice (CD1, Charles River), weighing 20–22 g, were used throughout the study. The animals were housed 30 per Makrolon box (*L*: 40, *W*: 25, *H*: 18 cm), with free access to standard semi-synthetic laboratory diet and tap water ad libitum, under controlled environmental conditions (temperature: 22 ± 1 °C, 7 a.m. to 7 p.m. light–dark cycle). Mice were tested only once and sacrificed immediately thereafter by decapitation. To assess the antinociceptive effects of the opioids, the hot-plate test was used. Intracerebroventricular (i.c.v.) injections (10 μl) were performed in the left brain ventricle of manually immobilized mice with a Hamilton microsyringe (50 μl) connected to a needle (diameter 0.5 mm), as described by Haley and McCormick [19]. All drugs for i.c.v. administration were dissolved in saline.

The hot-plate test was performed according to the method of Eddy and Leimbach [20]. A transparent plastic cylinder (14 cm diameter, 20 cm height) was used to confine the mouse on the heated (55 ± 0.5 °C) surface of the plate. The animals were placed on the hot-plate after i.c.v. injection of saline or peptides and the latencies to paw licking, rearing and jumping were measured. A cut-off time of 240 s was used to avoid tissue injury.

To evaluate the hot-plate test responses detailed below, the control latencies (t_0) and test latencies (t_1) were determined after injection of saline and a peptide, respectively. The percentage of the maximal possible effect (%MPE) was calculated as $\%MPE = (t_1 - t_0)/(t_2 - t_0) \times 100$, where the cut-off time (t_2) was 240 s.

The median antinociceptive dose (ED_{50}) was calculated according to the method described elsewhere [21].

2.4. Measurement of locomotor activity

Locomotor activity was assessed automatically in a Digiscan actimeter (Omnitech Electronics Inc., Columbus, OH, USA), which monitored horizontal displacements and vertical movements. The animals were injected i.c.v. with saline or peptides and placed individually in 20 cm × 20 cm × 30 cm compartments, in a dimly illuminated and quiet room. The responses were expressed as a total number of beams crossed by mouse during three consecutive periods of 10 min each.

Table 1 – Physicochemical data of the cyclic endomorphin-2 and morphiceptin analogs

Peptide no.	Sequence	HPLC t_R[a] (min)	FAB-MS			Purity (%)
			Formula	MW	$[M + H]^+$	
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	3.81	$C_{32}H_{37}N_5O_5$	571	572	97
2	Tyr-c(Lys-Phe-Phe-Asp)-NH_2	6.53	$C_{37}H_{45}N_7O_7$	699	700	98
3	Tyr-c(Asp-Phe-Phe-Lys)-NH_2	6.86	$C_{37}H_{45}N_7O_7$	699	700	98
4	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	3.95	$C_{28}H_{35}N_5O_5$	521	522	97
5	Tyr-c(Lys-Phe-D-Pro-Asp)-NH_2	5.92	$C_{33}H_{43}N_7O_7$	649	650	96
6	Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2	6.31	$C_{33}H_{43}N_7O_7$	649	650	98

[a] HPLC elution on a Vydac C_{18} column (1 cm × 25 cm) using the solvent system of 0.1% TFA in water (A) and 80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 20–90% (B) over 25 min.

2.5. *Statistical analysis*

The data are expressed as mean ± S.E.M. Differences between groups ($n = 10$) were assessed by one-way analysis of variance (one-way ANOVA) and a post hoc multiple comparison Student Newman–Keuls test. Antagonist effects of naloxone in the combination experiments were analyzed using two-way analysis of variance (ANOVA) and a post hoc multiple comparison Student Newman–Keuls test was used for multiple comparisons between groups. A probability level of 0.05 or smaller was used to indicate statistical significance.

3. Results

Two highly selective μ-opioid receptor ligands, endomorphin-2 and morphiceptin, have been chosen as parent compounds for the design of some conformationally constrained analogs. Cyclic pentapeptide analogs of endomorphin-2 and morphiceptin of the type Tyr-X-Phe-Phe-Y-NH_2 and Tyr-X-Phe-D-Pro-Y-NH_2 (X = Lys or Asp and Y = Lys or Asp), respectively, were synthesized (Table 1). Cyclization was carried out between side-chain amino and carboxy groups of diamino and dicarboxy amino acids introduced in positions 2 and 5 of the sequence, when the protected peptide was still bound to the resin. In the case of morphiceptin analogs, Pro in position 4 was replaced by D-Pro, as [D-Pro[4]]morphiceptin has greatly improved μ binding affinity, compared to morphiceptin [22].

Antinociceptive profile of the new analogs was studied in the hot-plate test in mice after i.c.v. administration (Table 2). In case of both, endomorphin-2 and morphiceptin cyclic analogs, substitution of Asp residue instead of Pro in position 2 and Lys in position 5 produced analogs with extremely improved antinociceptive activity compared to the parent peptides. Analogs with the opposite direction of the amide bond between amino acids in positions 2 and 5 were either inactive or showed decreased activity compared to the parent peptides.

For endomorphin-2, morphiceptin and the most potent Asp[2]-analogs **3** and **6** dose–response curves were obtained (Fig. 1). The ED_{50} values are shown in Table 3. Antinociceptive effect of **3** and **6** was dose-dependent and was much higher than that of the parent compounds. Fig. 2 shows the time-course of the antinociceptive effect of i.c.v.-administered endomorphin-2 and the most potent analogs, **3** and **6**. The hot-plate responses were measured 5, 10, 20, 30, 45 and 60 min after injection. The antinociceptive effect reached its peaks 5–10 min after injection, slowly declined, and returned to the pre-injection level 45–60 min after administration of endomorphin-2 and over 60 min after injection of **3** and **6**. Additionally, naloxone (5 μg, i.c.v.) was used to confirm that the action of new endomorphin-2 analogs was mediated through the opioid receptors. As shown in Fig. 3, the analgesic effect of **3** and **6** was antagonized by concomitant administration of naloxone.

The μ-, δ- and κ-binding affinities against [^{3}H]DAMGO, [^{3}H]DLT and [^{3}H]U-69593, respectively, are shown in Table 4. Surprisingly, the cyclic analogs of endomorphin-2 and morphiceptin showed only weak affinity at μ-opioid receptor and did not bind to the δ- and κ-sites.

The influence of the cyclic analogs on the locomotor activity was examined in mice after i.c.v. administration during three consecutive periods of 10 min each (Fig. 4). No

Table 2 – Antinociceptive effect of cyclic endomorphin-2 and morphiceptin analogs in the mouse hot-plate test after i.c.v. injection (10 μg/animal)

Peptide no.	Sequence	Latencies (%MPE) to					
		Paw licking	Relative efficacy	Rearing	Relative efficacy	Jumping	Relative efficacy
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	15.25 ± 4.51	1.00	27.35 ± 4.29	1.00	72.11 ± 5.62	1.00
2	Tyr-c(Lys-Phe-Phe-Asp)-NH_2	2.23 ± 0.41	0.15	7.52 ± 1.44	0.27	24.16 ± 2.62	0.34
3	Tyr-c(Asp-Phe-Phe-Lys)-NH_2	80.83 ± 6.66	5.30	93.81 ± 3.26	3.42	100%[a]	–
4	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	11.55 ± 4.18	1.00	33.56 ± 6.50	1.00	75.95 ± 8.19	1.00
5	Tyr-c(Lys-Phe-D-Pro-Asp)-NH_2	0.77 ± 0.04	0.07	0.85 ± 0.07	0.03	4.59 ± 0.45	0.06
6	Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2	100%[a]	–	100%[a]	–	100%[a]	–

[a] Percentage of mice with latency ≥240 s (cut-off time).

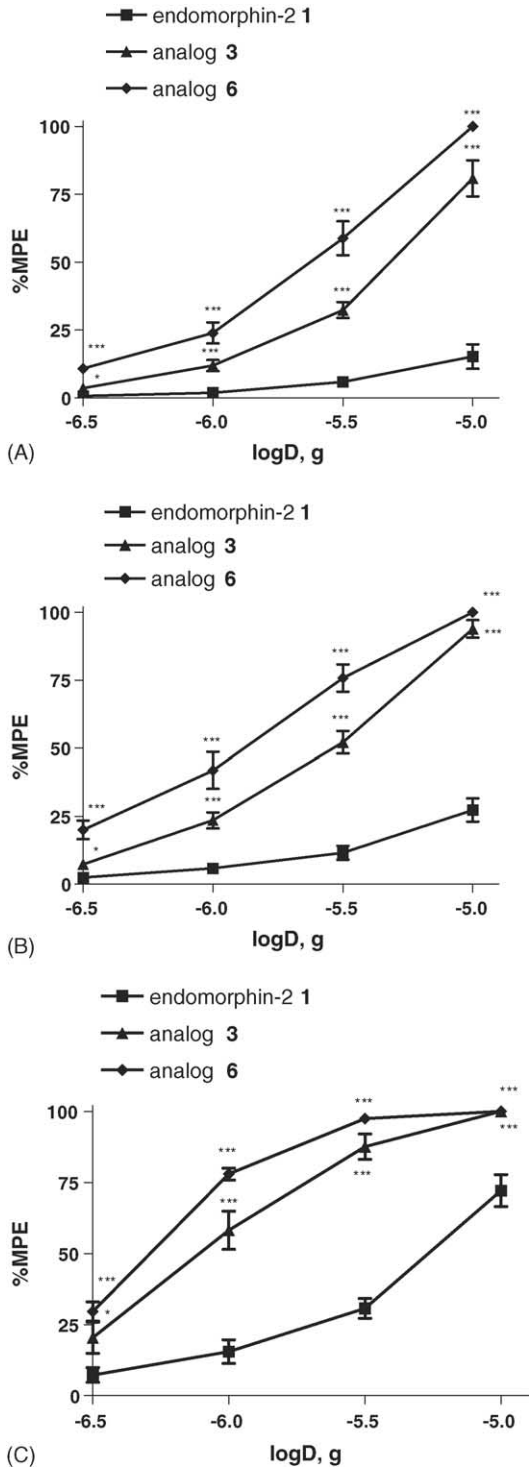

Fig. 1 – Dose–response curves for the hot-plate inhibition of paw licking (A), rearing (B) and jumping (C) induced by i.c.v. injection of endomorphin-2 1, Tyr-c(Asp-Phe-Phe-Lys)-NH_2 3 and Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2 6. The data represent mean ± S.E.M. of 10 mice per group. Statistical significance used one-way ANOVA and a post hoc multiple comparison Student Newman–Keuls test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from endomorphin-2 treated animals).

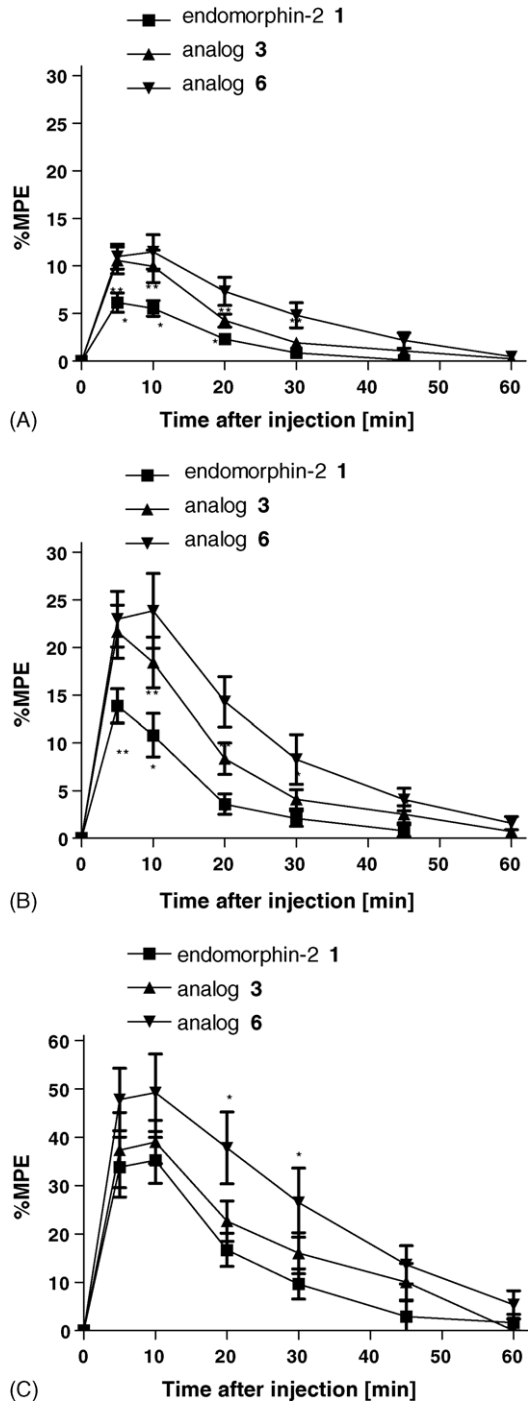

Fig. 2 – Time-course of the changes in i.c.v. endomorphin-2 1 (3 μg), Tyr-c(Asp-Phe-Phe-Lys)-NH_2 3 (1 μg) and Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2 6 (0.3 μg)-induced hot-plate inhibition of paw licking (A), rearing (B) and jumping (C). The data represent mean ± S.E.M. of 10 mice per group. Statistical significance used one-way ANOVA and a post hoc multiple comparison Student Newman–Keuls test ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from endomorphin-2 treated animals).

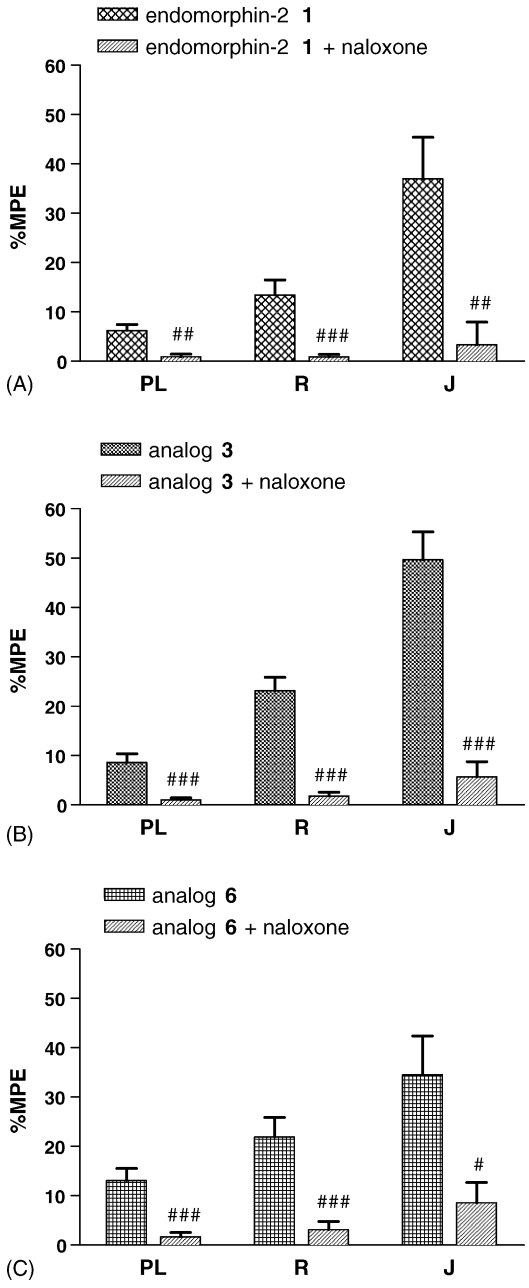

Fig. 3 – Comparison of the antagonist properties of naloxone (5 μg/animal, i.c.v.) on (A) endomorphin-2 1 (3 μg/animal, i.c.v.), (B) Tyr-c(Asp-Phe-Phe-Lys)-NH_2 3 (1 μg/animal, i.c.v.) and (C) Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2 6 (0.3 μg/animal, i.c.v.)-induced analgesia in the hot-plate test in mice. PL, paw licking; R, rearing; J, jumping. The data represent mean ± S.E.M. of 10 mice per group. A two-way ANOVA analysis revealed a significant interaction between: naloxone and endomorphin-2: $F(1,36) = 12.640$; $^{\#\#}p < 0.01$ (for paw licking), $F(1,36) = 13.840$; $^{\#\#\#}p < 0.001$ (for rearing), $F(1,36) = 7.56$; $^{\#\#}p < 0.01$ (for jumping); between naloxone and analog 3: $F(1,36) = 15.508$; $^{\#\#\#}p < 0.001$ (for paw licking), $F(1,36) = 49.860$; $^{\#\#\#}p < 0.001$ (for rearing), $F(1,36) = 25.005$; $^{\#\#\#}p < 0.001$ (for jumping) and between naloxone and analog 6: $F(1,36) = 17.522$; $^{\#\#\#}p < 0.001$ (for paw licking), $F(1,36) = 16.916$; $^{\#\#\#}p < 0.001$ (for rearing), $F(1,36) = 4.650$; $^{\#}p < 0.05$ (for jumping).

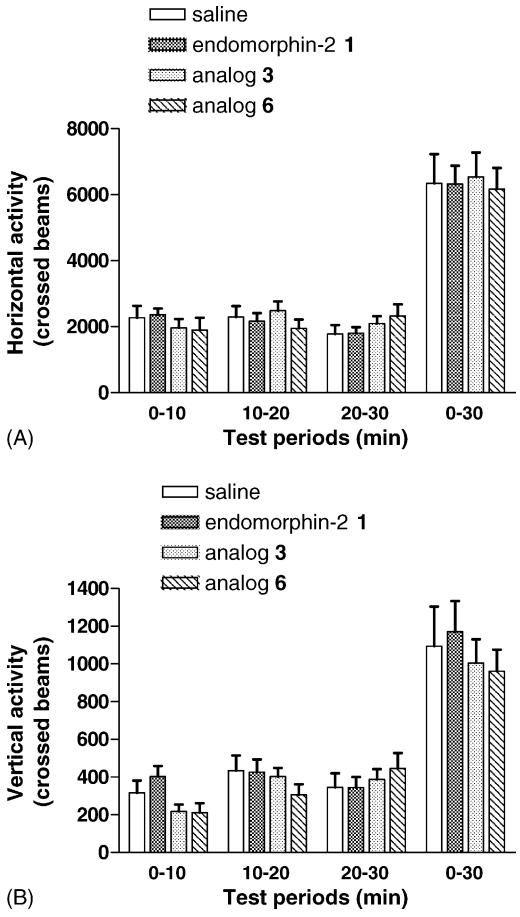

Fig. 4 – Effect of endomorphin-2 1 (3 μg), Tyr-c(Asp-Phe-Phe-Lys)-NH_2 3 (1 μg) and Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2 6 (0.3 μg), on locomotor activity. Mice were injected i.c.v. with saline or peptides and placed in the actimeters immediately after the injection. Horizontal (A) and vertical (B) components of locomotor activity were measured for three consecutive periods of 10 min. The data represent mean ± S.E.M. of 10 mice per group.

significant effect compared with control (saline) was observed for cyclic peptides **3** and **6**.

To explain the high antinociceptive activity of **3** and **6** the hot-plate test was performed with these peptides administered together with the selective κ-antagonist nor-BNI (Fig. 5). Nor-BNI only slightly inhibited endomorphin-2-induced analgesia, but almost completely reversed antinociception produced by **3** and **6**.

4. Discussion

Endomorphin-2 and morphiceptin have been shown to displace [^{3}H]DAMGO in the binding assay, which is consistent with the action of μ-opioid receptor agonists [23]. In behavioral experiments, i.c.v. administration of the μ-selective opioid peptides produces potent analgesia, which is blocked by the concomitant treatment with a non-specific opioid antagonist,

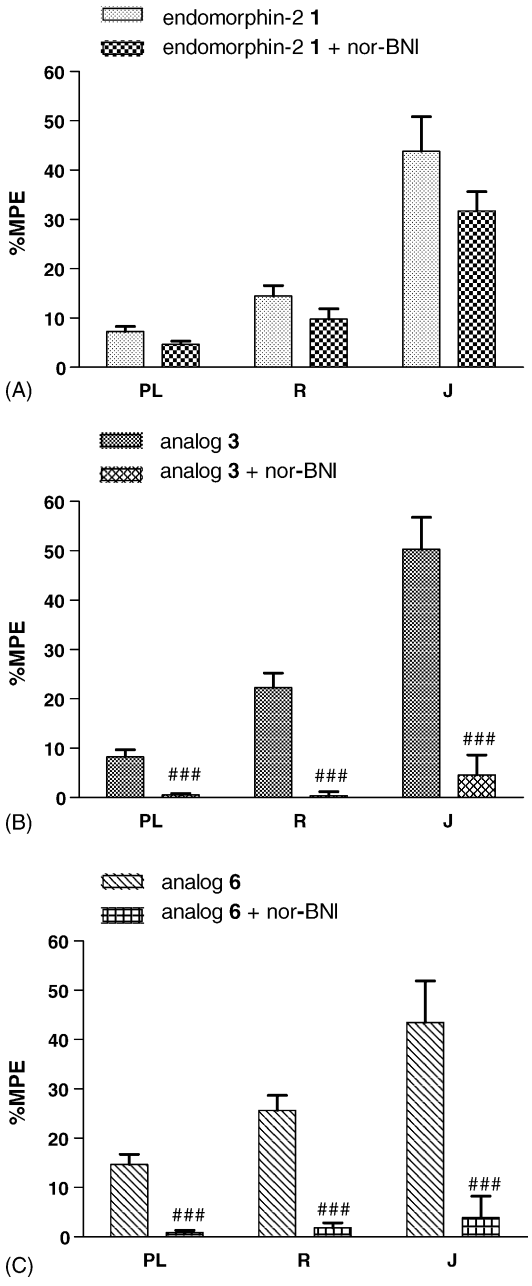

Fig. 5 – Comparison of the antagonist properties of nor-BNI (10 μg/animal, i.c.v.) on (A) endomorphin-2 1 (3 μg/animal, i.c.v.), (B) Tyr-c(Asp-Phe-Phe-Lys)-NH_2 3 (1 μg/animal, i.c.v.) and (C) Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2 6 (0.3 μg/animal, i.c.v.)-induced analgesia in the hot-plate test in mice. PL, paw licking; R, rearing; J, jumping. The data represent mean ± S.E.M. of 10 mice per group. A two-way ANOVA analysis revealed a significant interaction between nor-BNI and analog 3: $F(1,34) = 23.618$; $^{###}p < 0.001$ (for paw licking), $F(1,34) = 40.427$; $^{###}p < 0.001$ (for rearing), $F(1,34) = 36.193$; $^{###}p < 0.001$ (for jumping) and between nor-BNI and analog 6: $F(1,34) = 38.303$; $^{###}p < 0.001$ (for paw licking), $F(1,34) = 43.877$; $^{###}p < 0.001$ (for rearing), $F(1,34) = 13.063$; $^{###}p < 0.001$ (for jumping).

naloxone or a selective μ-opioid receptor antagonist, β-funaltrexamine [24]. In the μ-opioid receptor knock-out mice μ-opioid ligands do not produce any significant antinociceptive effects [25]. These findings strongly indicate that the μ-opioid receptor plays an essential role in mediating antinociceptive activity.

Two cyclic analogs of endomorphin-2 and morphiceptin synthesized in this study, Tyr-c(Asp-Phe-Phe-Lys)-NH_2 **3** and Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2 **6**, produced, after i.c.v. administration, a much stronger analgesic effect in the hot-plate test than endomorphin-2. This effect was dose-dependent and was reversed by naloxone, which indicates that it was mediated by the opioid receptors. The duration of the analgesic effect of the cyclic analogs was longer than the effect of endomorphin-2. However, none of the cyclic peptides showed any significant μ-, δ- or κ-receptor binding affinity.

To determine whether the increased latencies in the hot-plate test reflected true antinociception, or a general lack of responsiveness, a locomotor behavioral study, which does not contain any antinociceptive component, was performed for new cyclic analogs **3** and **6**. This test proved that **3** and **6** had no effect on the locomotor activity in mice; neither the muscles nor the central nervous system were affected.

In the earlier studies Tseng [26] showed that antinociception induced by endomorphin-2, but not endomorphin-1, was partially blocked by the pretreatment with the κ-receptor antagonist nor-BNI, indicating that analgesia produced by endomorphin-2 was elicited, in part, by the stimulation of the κ-receptor. It is most likely that endomorphin-2 produced its antinociception by the release of dynorphin A, which subsequently acted on the κ-receptor. This was evidenced by the finding that the pretreatment of mice with an antiserum against dynorphin A, which bound the released dynorphin A, partially attenuated the antinociception induced by endomorphin-2.

In our study, very high antinociceptive activity of the cyclic analogs **3** and **6** was inconsistent with their moderate μ-receptor binding affinity. The obtained data strongly indicate that the cyclic peptides elicited their analgesic effects partially through the μ-receptors and partially by the release of dynorphin A, which acted through the κ-opioid receptor.

A less probable possibility, which, however, should be also taken into consideration is that **3** and **6** could be opioid peptide prodrugs. They could be pharmacologically inactive derivatives, which are converted into an active drug within the body through some enzymatic or non-enzymatic reactions. This hypothesis needs more detailed studies in the future.

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Table 3 – ED_{50} values for endomorphin-2 and its most potent cyclic analogs

Peptide no.	Sequence	ED_{50} (μg)		
		Paw licking	Rearing	Jumping
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	>1000	946 ± 122	1.83 ± 0.15
3	Tyr-c(Asp-Phe-Phe-Lys)-NH_2	3.91 ± 0.43	1.73 ± 0.22	0.45 ± 0.03
6	Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2	1.52 ± 0.11	0.87 ± 0.07	0.22 ± 0.01

Table 4 – Opioid receptor binding affinities of cyclic endomorphin-2 and morphiceptin analogs

Peptide no.	Sequence	K_i ± S.E.M. (nM)		
		μ[a]	δ[b]	κ[c]
1	Tyr-Pro-Phe-Phe-NH_2 (endomorphin-2)	3.14 ± 0.69	>1000	>1000
2	Tyr-c(Lys-Phe-Phe-Asp)-NH_2	>1000	>1000	>1000
3	Tyr-c(Asp-Phe-Phe-Lys)-NH_2	289 ± 45	>1000	>1000
4	Tyr-Pro-Phe-Pro-NH_2 (morphiceptin)	68.6 ± 12.2	>1000	>1000
5	Tyr-c(Lys-Phe-D-Pro-Asp)-NH_2	>1000	>1000	>1000
6	Tyr-c(Asp-Phe-D-Pro-Lys)-NH_2	514 ± 98	>1000	>1000
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (DLT)	>1000	0.78 ± 0.55	>1000
	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-NH_2 [dynorphin(1-13)-NH_2]	>1000	>1000	0.029 ± 0.011

[a] Displacement of [^{3}H]DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol).
[b] Displacement of [^{3}H]DLT (Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2).
[c] Displacement of [^{3}H]U-69593.

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