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Original article

A structure–activity study of nociceptin-(1–13)-peptide amide. Synthesis of analogues substituted in positions 0, 1, 3, 4 and 10

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Abstract

A series of analogues of nociceptin, Noc(1–13)NH_2 (an agonist at the ORL1 receptor) was synthesized with following modifications: (1) N-terminal extension with Arg^0; (2) replacement of Gly^3 by basic or polar amino acids—Arg, Asn, Lys(For) or deletion; (3) exchange of Phe^1 or Phe^4 by $Phe(NO_2)$; (4) substitution of Ser^{10} with D-Ser, Pro, D-Pro. The analogs were synthesized by solid-phase methodology using Fmoc-amino acid pentafluorophenyl esters. The affinity for the ORL1 and for the κ, μ and δ-opioid receptors was investigated by radioligand binding assay and bioactivity by a mouse vas deferens (MVD) assay. The addition of the amino acid residue Arg to the N-terminal enhances the opioid receptor affinity of Noc(1–13)NH_2 while retaining ORL1 receptor affinity at a moderate level. The replacement of Gly in position 3 by the basic or polar amino acids—Arg, Asn, Lys(For) or its deletion led to inactive analogues. The replacement of Ser in position 10 by its D-isomer, Pro and D-Pro resulted in a series of analogues with the following order of activity: $Ser^{10} > D\text{-}Ser^{10} > Pro^{10} > D\text{-}Pro^{10}$. In [D-$Ser^{10}$]Noc(1–13)$NH_2$, introduction of an additional $Phe(NO_2)^4$ led to a >60-fold increase of ORL1 affinity, completely attenuating the loss of affinity brought about by Ser^{10}. In other analogues, introduction of $Phe(NO_2)^4$ did not change the magnitude of ORL1 binding significantly. Generally, while modifications in position 3 frequently led to a loss of most or all bioactivity, modifications in position 0 (Arg^0) or 4 ($Phe(NO_2)^4$) and 10 (D-Ser^{10}, Pro^{10}) are tolerated.

Keywords: Nociceptin analogues; Peptide synthesis; Binding assay; MVD biossay

1. Introduction

The heptadecapeptide–nociceptin (also named orphanin F/Q, whose sequence is Phe–Gly–Gly–Phe–Thr^5–Gly–Ala–Arg–Lys–Ser^{10}–Ala–Arg–Lys–Leu–Ala^{15}–Asn–Gln is the naturally occurring ligand of the ORL1 receptor [1,2]. Nociceptin shows structural similarity to the opioid peptide dynorphin A (Dyn A), the endogenous ligand of the κ opioid receptor (Tyr–Gly–Gly–Phe–Leu^5–Arg–Arg–Ile–Arg–Pro^{10}–Lys–Leu–Lys–Trp–Asp^{15}–Asn–Gln). Both have the same length (17 amino acids) and contain six identical amino acids. Both peptides contain four to five basic amino acid residues. However, those amino acids are distributed differently at the C-terminal parts of the molecules. The ORL1 receptor also exhibits a significant degree of sequence identity with opioid receptors, particularly the κ opioid receptor. Despite the structural homology of nociceptin and dynorphin A, they do not activate each other's receptors [2]. Structure–activity relationship studies on nociceptin showed, that Phe^1 could be changed to a tyrosine without any change in binding affinity or bioactivity in vitro [3]. The corresponding modification of Dyn A(1–11)NH_2—substituting the Tyr^1 in by

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Phe—leads to a ca. 20-fold loss in receptor binding affinity and to a >200-fold potency drop in the GPI assay [4]. Alanine scans on nociceptin [5] and on dynorphin [6] have been carried out. In both peptides, the N-terminus plays a more critical role in binding than the C-terminus and the presence of aromatic residues in positions 1 and 4 is essential. In nociceptin, replacement of Gly^2 or Arg^8 by Ala also leads to a drastic reduction of ORL1 affinity.

The present investigation was undertaken to explore the role of the basic and polar amino acid residues in ligand/receptor selectivity of nociceptin analogs. Results from structure–activity studies on nociceptin have demonstrated that it can be truncated to the 13-peptide amide without significant loss of potency [7]. Therefore, we have used Noc(1–13)NH_2 as a template to examine structure–activity relationships of nociceptin analogs in this work.

A series of analogs of Noc(1–13)NH_2 was synthesized with the following modifications: (1) N-terminal extension with Arg^0; (2) replacement of Gly^3 by the basic or polar amino acids Arg, Asn or Lys(For) or deletion; (3) exchange of Phe^4 for Phe(NO_2); (4) Ser^{10} was substituted with D-Ser, Pro, D-Pro.

The biological activity of Noc(1–13)NH_2 and its analogs was estimated by radioligand binding assay and mouse vas deferens (MVD) assay. Nociceptin and its analogs inhibit the electrically stimulated contraction of this preparation [8]. The effect is not antagonized by naloxone [7] suggesting that it is mediated by the ORL1 and not by an opioid receptor. The MVD was therefore chosen as a functional assay to assess the potency of our ORL1 ligands.

1.1. Abbreviations

Symbols and abbreviations are in accord with the recommendations of the IUPAC–IUB Joint Commission on Biochemical Nomenclature (*Biochem. J.* (1984) **219**, 345–373). All optically active amino acids are of the L variety unless otherwise stated. Abbreviations used are: AcOH, acetic acid; DADLE, [D-Ala^2, D-Leu^5]-enkephalin; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; Dyn, dynorphin; For, formyl; GPI, guinea pig ileum; HATU, (*O*-(7-azabenzotriazol-l-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazol; MeCN, acetonitrile; MeOH, methanol; Mtr, 4-methoxy-2,3,6-trimethylbenzenesulphonyl; MVD, mouse vas deferens; *n*BuOH, *n*-butanol; Noc, nociceptin; OR, opiate receptor; ORL1 receptor, opioid receptor-like 1 receptor; PAL, peptide amide linker; Pbf, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl; PD-MS, plasma desorption mass spectrometry; PEG, polyethylene glycol; Pfp, pentafluorophenyl; Phe(p-NO_2), 4-nitro-L-phenylalanine; PS, polystyrene; RP-HPLC, reverse-phase high performance liquid chromatography; ^{t}Bu, *tert*-butyl; TFA, trifluoroacetic acid; TLC, thin layer chromatography; Trt, trityl.

2. Chemistry

The peptides were synthesized by automated solid phase peptide synthesis using the standard base labile 9-fluorenylmethoxycarbonyl (Fmoc) synthetic strategy on PEG-PS (polyethylene glycol polystyrene) supports.

The side chains were protected with Trt for Asn, Boc for Lys, Mtr or Pbf for Arg, and ^{t}Bu in the case of Thr and Ser. The pentafluorophenyl esters of Fmoc-amino acids were coupled to the growing peptide chain and HOBt was added to the OPfp esters to maximize the kinetics of the reaction. Amino acid residues of D-Pro, D-Ser, Phe(p-NO_2) and Lys(For) were coupled using HATU and DIEA. The Fmoc protecting group was removed with 20% (v/v) piperidine in DMF. The peptides were deprotected and cleaved from the PEG-PS support using concentrated TFA in the presence of scavengers.

Purification was achieved by preparative RP-HPLC on a Nucleosil C_{18} (2×25 cm) column employing gradients of H_2O/CH_3CN containing 0.1% TFA. Homogeneity of the purified peptides was assessed by analytical HPLC and TLC. Structure identification was achieved by amino acid analysis and PD-MS (Table 1).

3. Results and discussion

The peptides were evaluated for ORL1 receptor affinity and opioid receptor affinity (μ,δ,κ-receptors) by radioligand binding assay. In vitro activity of the peptides was determined by MVD assay as described recently by Guerrini et al. [7]. The results are presented in Tables 2 and 3.

3.1. Modifications of position 0

The concept of N-terminal extension has previously been applied to opioids. The addition of Arg to the N-terminus of Met-enkephalin practically did not change the activity in the guinea pig ileum [9] but N-terminal extension by Lys led to a number of enkephalin analogues with increased potency in the GPI [10].

Increasing the size of the Noc(1–13)NH_2 (**1**) by addition of an Arg residue to the N-terminal endows the nociceptin molecule with some opioid properties while retaining ORL1 receptor affinity at a moderate level. Thus, [Arg^0]Noc(1–13)NH_2 (**19**) has a sixfold higher κ-receptor affinity and shows a moderate ORL1-affinity in comparison to the parent compound **1**. With $K_{i\kappa}/K_{iORL1} = 62$, the selectivity of **19** is 150 times smaller

Table 1
Abbreviated names, structure and analytical properties of nociceptin(1–13)-NH_2 and its analogues

No	Substituted amino acid residues in nociceptin(1–13)-NH_2	Structure of compounds	PDMS MH^+		HPLC (k′) [a]
			Calculated	Found	
1		FGGFTGARKSARK-NH_2	1382.5	1382.4	4.07
2	[D-Ser^{10}]	FGGFTGARKsARK-NH_2	1382.5	1383.3	2.81
3	[Pro^{10}]	FGGFTGARKPARK-NH_2	1392.6	1392.8	4.12
4	[D-Pro^{10}]	FGGFTGARKpARK-NH_2	1392.6	1392.7	1.96
5	[Arg^3]	FGRFTGARKSARK-NH_2	1481.7	1482.2	3.31
6	[Arg^3,D-Ser^{10}]	FGRFTGARKsARK-NH_2	1481.7	1481.6	3.25
7	[Arg^3,Pro^{10}]	FGRFTGARKPARK-NH_2	1491.8	1491.7	3.85
8	[Arg^3,D-Pro^{10}]	FGRFTGARKpARK-NH_2	1491.8	1492.1	3.85
9	[Asn^3,D-Ser^{10}]	FGNFTGARKsARK-NH_2	1439.7	1439.9	3.78
10	[Asn^3,Pro^{10}]	FGNFTGARKPARK-NH_2	1449.7	1450.2	1.14
11	[Asn^3,D-Pro^{10}]	FGNFTGARKpARK-NH_2	1449.7	1449.8	1.57
12	[des G1y^3,Phe(p-NO_2)^4]	FGF(p-NO_2)TGARKSARK-NH_2	1370.5	1371.2	2.07
13	[des G1y^3,D-Ser^{10}]	FGFTGARKsARK-NH_2	1347.5 [b]	1349.1	1.06
14	[des Gly^3,Pro^{10}]	FGFTGARKPARK-NH_2	1357.6 [b]	1357.1	1.25
15	[des G1y^3,D-Pro^{10}]	FGFTGARKpARK-NH_2	1335.6	1335.4	1.71
16	[Lys(For)^3]	FGK(For)FTGARKSARK-NH_2	1481.7	1482.5	1.85
17	[Lys(For)^3,Phe(p-NO_2)^4]	FGK(For)F(p-NO_2)TGARKSARK-NH_2-	1526.7	1526.9	0.33
18	[Phe(p-NO_2)^1,Lys(For)^3, Phe(p-NO_2)^4]	F(p-NO_2)GK(For)F(p-NO_2) TGARKSARK-NH_2	1571.7	1571.5	1.88
19	[Arg^0]	RFGGFTGARKSARK-NH_2	1538.8	1537.3	4.00
20	[Arg^0,Arg^3]	RFGRFTGARKSARK-NH_2	1637.9	1638.2	2.62
21	[Phe(p-NO_2)^4]	FGGF(p-NO_2)TGARKSARK-NH_2	1427.6	1426	4.21
22	[Arg^0,D-Ser^{10}]	RFGGFTGARKsARK-NH_2	1538.8	1538	4.23
23	[Arg^0,Phe(p-NO_2)^4,D-Ser^{10}]	RFGGF(p-NO_2)TGARKSARK-NH_2	1583.8	1582.5	4.61
24	[Phe(p-NO_2)^4,D-Ser^{10}]	FGGF(p-NO_2)TGARKsARK-NH_2	1427.6	1426.6	4.43

[a] Capacity factor; s-D-isomer of Ser; p-D-isomer of Pro.
[b] Value in table is $M+Na^+$.

than the selectivity of Noc(1–13)NH_2 (**1**) ($K_{i\kappa}/K_{iORL1}$ = 9800).

Additional substitution of Ser^{10} by D-Ser gives [Arg^0, D-Ser^{10}]Noc(1-13)NH_2 (**22**) which also shows a 10-fold reduction of ORL1 affinity (K_i = 1.9 nM). Surprisingly, the loss of ORL1 affinity observed for D-Ser^{10} analog (**2**) (K_i = 15.3 nM) is partly attenuated by Arg^0. Likewise, [Arg^0,Arg^3]Noc(1–13)NH_2 (**20**) shows five times higher binding affinity at the ORL1 receptor than [Arg^3]Noc(1–13)NH_2 (**5**). However, the κ affinity of analog **22** is increased >40-fold and its μ affinity is increased fivefold relative to Noc(1–13)NH_2.

The trisubstituted analog [Arg^0, Phe(p-NO_2)^4, D-Ser^{10}]Noc(1–13)NH_2 ((**23**), K_i = 2.2 nM) shows decreased ORL1-affinity as compared to the disubstituted analog [Arg^0, D-Ser^{10}]Noc(1–13)NH_2 ((**24**), K_i = 0.23 nM).

3.2. Modifications of position 3

Substitution of the Gly^3 amino acid residue in enkephalins leads to a drop in potency, which in most cases is marked or complete. The introduction of L-amino acid residues (Ala or others) in place of Gly^2 likewise causes a marked drop in potency in most cases, but the substitution of D-amino acid residues instead of Gly^2 may cause a marked increase of potency in both GPI and MVD assays [11].

In contrast, in dynorphins modification by Ala-scanning at position 3 is better tolerated than modification in position 2 [12]. Thus, [Ala^3]Dyn A(1–13) retains about 35% of the potency in GPI assay, while the potency of [Ala^2]Dyn A(1–13) was negligible. As for the binding affinity to the κ opioid receptor, substitution of Dyn A(1–11)-NH_2 with Ala or D-Ala at position 3 resulted in increased κ selectivity without significant loss in affinity [13,14].

In an investigation of Ala-substituted nociceptin peptides (3), it has been shown that substitution of Gly^3 with Ala results in a more active compound than substitution of Gly in position 2 with Ala. Opposite results were obtained for the D-alanine-substituted nociceptin analogues: [D-Ala^2]nociceptin showed more affinity to the ORL1 receptor than [D-Ala^3]nociceptin [3].

In our work, Gly^3 was substituted by polar amino acids such as Arg, Asn, Lys(For). The analog [Arg^3]Noc(1–13)NH_2 (**5**) turned out to be ca. 8000-fold weaker than the parent compound Noc(1–13)NH_2 (**1**). The Lys(For)^3 analogue (**16**) also showed circa

Table 2
Radioligand binding affinities of nociceptin(1–13)-NH_2 analogues

No.	Position of substituted amino acid residues					K_i (nM)			
	0	1	3	4	10	ORL1 [a]	μ [b]	δ [b]	κ [b]
1						0.18	4730	> 1000	1770
2					D-Ser^{10}	15.3	43%	8%	71%
3					Pro^{10}	43	42%	15%	56%
4					D-Pro^{10}	104	35%	7%	47%
5			Arg^3			1400	35%	0%	75%
6			Arg^3		D-Ser^{10}	940	28%	0%	76%
7			Arg^3		Pro^{10}	1493	29%	13%	78%
8			Arg^3		D-Pro^{10}	64%	12%	0%	70%
9			Asn^3		D-Ser^{10}	30%	12%	0%	74%
10			Asn^3		Pro^{10}	58%	41%	0%	27%
11			Asn^3		D-Pro^{10}	68%	28%	0%	47%
12			–^{3}	Phe(NO$_2$)^4		46	12%	4%	59%
13			–^{3}		D-Ser^{10}	53%	17%	0%	61%
14			–^{3}		Pro^{10}	44%	10%	0%	32%
15			–^{3}		D-Pro^{10}	19%	7%	0%	48%
16			Lys(For)^3			745	21%	0%	53%
17			Lys(For)^3	Phe(NO$_2$)^4		160	47%	11%	76%
18		Phe(NO$_2$)^1	Lys(For)^3	Phe(NO$_2$)^4		425	28%	1%	77%
19	Arg^0					4.8	3700	> 10,000	300
20	Arg^0		Arg^3			240	31%	0%	79%
21				Phe(NO$_2$)^4		0.20	1770	> 1000	1250
22	Arg^0				D-Ser^{10}	1.9	930	> 1000	41
23	Arg^0			Phe(NO$_2$)^4	D-Ser^{10}	2.2	3600	> 1000	670
24				Phe(NO$_2$)^4	D-Ser^{10}	0.23	2200	> 1000	630

K_i (nM) or % ligand displacement at $c = 10$ μM.
[a] Reference ligand: [^{3}H] nociceptin.
[b] Reference ligand: [^{3}H] diprenorphine.

Table 3
Functional assays of nociceptin and nociceptin(1-13)NH_2 analogues using the electrically stimulated MVD [a]

No.	Position of substituted amino acid residues				EC50	pEC50 [c]	I_{max}
	0	3	4	10	(nM) [b]		(%) [d]
2				D-Ser^{10}	8100	5.09 ± 0.13	48 ± 6
3				Pro^{10}	7600	5.12 ± 0.23	41 ± 5
4				D-Pro^{10}	62	7.17 ± 0.08	60 ± 6
5		Arg^3			760	6.12 ± 0.21	54 ± 3
6		Arg^3		D-Ser^{10}	6500	5.19 ± 0.19	55 ± 7
7		Arg^3		Pro^{10}	5100	5.29 ± 0.14	50 ± 6
8		Arg^3		D-Pro^{10}	27,000	4.57 ± 0.09	46 ± 3
9		Asn^3		D-Ser^{10}	910	6.04 ± 0.07	51 ± 4
10		Asn^3		Pro^{10}	28,000	4.55 ± 0.11	48 ± 6
11		Asn^3		D-Pro^{10}	100,000	4.00 ± 0.12	43 ± 8
12		–^{3}	Phe(NO$_2$)^4		1700	5.77 ± 0.19	56 ± 7
13		–^{3}		D-Ser^{10}	62,000	4.21 ± 0.09	43 ± 1
14		–^{3}		Pro^{10}	85,000	4.07 ± 0.1	40 ± 5
15		–^{3}		D-Pro^{10}	3600	5.44 ± 0.07	51 ± 6
16		Lys(For)^3			690,000	3.16 ± 0.22	30 ± 5
17		Lys(For)^3	Phe(NO$_2$)^4		43,000	4.37 ± 0.08	47 ± 7
18	Phe(NO$_2$)^1	Lys(For)^3	Phe(NO$_2$)^4		2400	5.62 ± 0.11	51 ± 3
20	Arg^0	Arg^3			71,000	4.15 ± 0.23	46 ± 1
Nociceptin					42	7.38 ± 0.14	76 ± 9
DADLE					160	6.80 ± 0.16	65 ± 4

[a] Cf. Section 4.2.2 for assay conditions.
[b] Molar concentration of an agonist which produces 50% of maximum inhibition of electrically induced twitches.
[c] Negative decadic logarithm of EC_{50}.
[d] The maximal effect of an agonist expressed as percent inhibition of electrically induced twitches.

4000-fold reduction of ORL1 affinity. All analogues containing Asn in position 3 were inactive.

Nociceptin analogues with double replacements—combination of polar amino acids in position 3 and D-Ser, D-Pro or Pro in position 10 were also synthesized. Almost all of compounds **6**–**11** showed drastic reduction of affinity to both ORL1 and opioid receptors.

As has been demonstrated earlier, a single deletion of Gly in position 3 in the native sequence of enkephalin reduces opiate activity drastically. However, the potency losses resulting from the elimination of Gly^3 can be fully attenuated by substitution with D-Ala at position 2 [15]. Almost the same results were observed here. While [desGly^3]Noc(1–13)NH_2 was found to be completely inactive [7], the second modification, namely, the insertion of Phe(p-NO_2)^4, instead of Phe^4, results in a compound [desGly^3, Phe(p-NO_2)^4]Noc(1–13)NH_2 (**12**), which showed moderate affinity to the ORL1 receptor ($K_i = 46$ nM).

The combination of Gly^3 elimination and replacement of the Ser amino acid residue by D-Ser, D-Pro or Pro in position 10 led to the analogs **13**–**15**, which were inactive at all receptors.

3.3. Modifications of position 4

Replacement of Phe^4 by Phe(p-NO_2)^4 is a modification which has led to highly active enkephalin analogues [16,17]. In dynorphins, the structural requirements concerning Phe(p-NO_2)^4 at position 4 probably are different for the native sequence as compared to analogs with multiple changes in the molecule. Introduction of Phe(p-NO_2) in position 4 of Dyn(1–13) was shown to produce a fourfold drop in potency on the GPI [16]. However, in the case of dynorphin analogues with multiple changes in the molecule, Phe(p-NO_2)^4 increases κ- and μ-affinity. For example, [Me-Tyr^1, Phe(p-NO_2)^4, D-Leu^5] Dyn(1–13)-NH_2 demonstrated threefold higher μ-receptor affinity and twofold higher κ-receptor affinity as compared to [Me-Tyr^1, D-Leu^5]Dyn(1–13)-NH_2. Its analgesic activity also was increased and was comparable with that of morphine [18].

In this study, the analogue [Phe(p-NO_2)^4]Noc(1–13)NH_2 (**21**) was synthesized. This modification did not affect the ORL1 receptor activity of analogue **21** and keeps it at the same high level of potency as in Noc(1–13)NH_2 (**1**). However, the μ-affinity of analogue **21** is more than doubled. This suggests that the electron-withdrawing substituent endows the native nociceptin sequence with more opioid properties.

The doubly substituted nociceptin analogue [Phe(p-NO_2)^4, D-Ser^{10}]Noc(1–13)NH_2 (**24**) also retains a high level of potency at the ORL1 receptor ($K_i = 0.23$ nM), but shows about a twofold increase in κ-affinity in comparison to [Phe(p-NO_2)^4]Noc(1–13)NH_2 (**21**).

In this work we show that the potency losses at the ORL1 receptor of nociceptin analogues resulting from the substitution of Ser^{10} to D-Ser^{10} can be fully compensated by incorporation of Phe(p-NO_2)^4 instead of Phe in position 4. While D-Ser in position 10 (analogue **2**) leads to a reduction of ORL1 receptor affinity by two orders of magnitude in comparison to Noc(1–13)NH_2 (**1**), the second substitution of Phe^4 to Phe(p-NO_2)^4 completely restores the ORL1 receptor affinity. [Phe(p-N0$_2$)^4, D-Ser^{10}]Noc(1–13)NH_2 (**24**) shows almost the same affinity ($K_i = 0.23$ nM) to the ORL1 receptor as Noc(1–13)NH_2 (**1**) ($K_i = 0.18$ nM). Though Phe(NO_2)^4 does not increase the ORLI affinity of the natural sequence, the increase observed in the D-Ser^{10} analogue is about 60-fold. Exploration of these observations is highly interesting, as it provides an approach to create lead compounds for further enhancement ORLI receptor potency and selectivity.

Extension of this molecule from the N-terminus by Arg^0 gives [Arg^0, Phe(p-NO_2)^4, D-Ser^{10}]Noc(1–13)NH_2 (**23**). This modification causes a decrease of ORL1 receptor affinity by one order of magnitude, but retains almost the same κ-receptor affinity as compound **24**. For this reason, compound **23** has 9 times smaller selectivity for the ORLl receptor than [Phe(p-NO_2)^4, D-Ser^{10}]Noc(1–13)NH_2 (**24**) compound.

3.4. Modifications of position 10

It has been shown for dynorphins that the replacement of Pro in position 10 by its D-isomer resulted in [D-Pro^{10}]Dyn(1–11), which shows both high affinity and selectivity for the κ opioid receptor [19].

Similar modifications of nociceptin turned out to be less successful. Replacement of the amino acid residue Ser^{10} by its D-isomer resulted in [D-Ser^{10}]Noc(1–13)NH_2 (**2**), which displays 100-fold weaker ORL1 affinity than the parent peptide Noc(1–13)NH_2 (**1**). Substitution with Pro and D-Pro at this position led to further reduction of ORL1 affinity. The ORL1 affinity of the analogues substituted in position 10 followed to the order:

Ser^{10}($K_i = 0.18$ nM) > D-Ser^{10} ($K_i = 15.3$ nM) > Pro^{10} ($K_i = 43$ nM) > D-Pro^{10} ($K_i = 104$ nM).

Substitution with D-amino acids or proline causes profound changes in the backbone conformation of a peptide, which do not seem to be favourable at this position. As mentioned above, the negative effect of D-Ser^{10} on ORL1 affinity can be compensated by an additional Phe(p-NO_2)^4. Of the other disubstituted analogues, only the [Arg^3, D-Ser^{10}] analogue (**6**) and the [Arg^3, Pro^{10}] analogue (**7**) retained some ORL1 affinity (ca. 1 μM). Combination of the aforementioned modifications of position 10 with Asn^3 (analogues **9**–**11**) or deletion of Gly^3 (analogues **13**–**15**) led to inactive compounds.

4. Experimental protocols

4.1. Chemistry

Fmoc-amino acid derivatives, supports and reagents were purchased from KEBO Lab (Stockholm) except for Fmoc-D-Ser, Fmoc-D-Pro, Fmoc-Lys(For), Fmoc-Phe(p-NO_2) which were purchased from Bachem (Switzerland). The reagents used in peptide synthesis were as follows: Fmoc-PAL-PEG-PS (for peptide amides), HOBt, HATU, DIEA, pentafluorophenyl esters of Fmoc-Ala, Fmoc-Arg(Mtr), Fmoc-Arg(Pbf), Fmoc-Gly, Fmoc-Lys(Boc), Fmoc-Ser(^{t}Bu), Fmoc-Thr(^{t}Bu), Fmoc-Phe, Fmoc-Pro, Fmoc-Asn(Trt) and Fmoc-protected amino acids of D-Ser, D-Pro, Lys(For), Phe(p-NO_2). HPLC-grade solvents DCM, DMF, MeCN and MeOH were used in synthesis and purification. Other reagents were reagent grade.

Syntheses of Noc(1–13)NH_2 and its analogues were performed by the solid phase method either manually (for unusual amino acids) or utilizing an automated synthesizer-MilliGen 9050 PepSynthesizer system using a standard Fmoc protocol on Fmoc-PAL-PEG-PS resin. Couplings of unusual amino acids were performed using HATU for the coupling of amino acid residues to the growing peptide resin in the presence of DIEA.

The synthesized analogues were purified by RP-HPLC and characterized by PD-mass spectrometry and amino acid analysis. The purity of the peptides was assessed by TLC (single spot in three different solvent systems) and HPLC (one single peak, UV detection at 280 and 220 nm). Thin layer chromatography of nociceptin analogues was performed on Silica gel 60F 254 received from Merck (Germany) in systems: nBuOH–pyridine–AcOH–H_2O (15:10:3:6); nBuOH–AcOH–H_2O (4:1:1); $CHC1_3$–MeOH–AcOH–H_2O (30:20:4:6). Chromatograms were visualized by UV irradiation, by spraying with ninhydrin solution or using chlorine–benzidine reagent. Amino acid analysis was performed in a Biocal BC-200 amino acid analyser after peptide hydrolysis in a sealed ampoule (24 h at 110 °C), using ninhydrin detection. Reversed phase HPLC was done using a Pharmacia LKB gradient HPLC system with a RSD 2140 detector (LC Controller LCC 2252, Pump 2248) and column (20 × 250 mm, 15 μm spherical particle size, C_{18}). The peptides were eluted using a binary system composed of 0.1% TFA in H_2O (solvent A) and 0.1% TFA in MeCN (solvent B). The following linear gradients were used: (1) 10–30% solvent B over 10 min; (2) 30–100% solvent B over 40 min. Mass spectroscopy was performed on an Applied Biosystems BIOION 20 plasma desorption mass spectrometer. Samples were applied on aluminised mylar foils, coated with electrosprayed nitrocellulose, by drying from EtOH–H_2O–TFA mixtures and removal of excess liquid by nitrogen.

4.1.1. Solid phase peptide synthesis of protected Noc(1–13)NH_2-resin

The resin (Fmoc-PAL-PEG-PS) 0.44 g (0.16 mmol g^{-1} resin substitution) was washed 5 × 20 mL of DMF and the synthesis carried out as follows: the deprotection of the resin was performed for 3 min and then 7 min in 20% piperidine in DMF (v/v) and then the resin washed with DMF (12 ×). The solution of the desired pentafluorophenylesters of Fmoc amino acid (4.0–fold excess) in DMF (0.2 M) in the presence of 1.5 equiv. of HOBt was reacted with the resin for 2 h; the resin was washed with DMF (12 ×). After completion of the peptide assembly and removal of the Fmoc group from the N-terminal residue, the resin was washed successively with DMF, DCM, MeOH and DCM, collected and dried overnight in vacuo.

4.1.2. Cleavage of Noc(1–13)NH_2 from the solid support

The dried protected peptide resin was treated with 2.5 mL of Reagent B (93% TFA, 2% water, 2% thioanisole and 3% ethanedithiol) under nitrogen for 4–4.5 h twice. The peptide was filtered from the resin and washed with 2 mL of TFA. The filtrate was concentrated under reduced pressure. The residue was triturated with cold ether, centrifuged, washed with cold ether and dried in vacuo overnight, yielding the crude peptide. The crude peptide was purified by RP-HPLC as described above to yield a white powder after lyophilization. The structure of peptide **1** was confirmed by the results of measurements of molecular weight and amino acid analysis, the purity characterized by analytical HPLC and TLC.

4.1.3. Synthesis of peptide analogues **3, 5, 7, 10, 14, 19, 20**

The peptides were synthesized and purified as described for compound **1**. The structure of the peptides, obtained after lyophilization as white powder, was confirmed by mass spectrometry and amino acid analysis. The purity was estimated by HPLC and TLC.

4.1.4. Synthesis of peptide analogues **2, 4, 6, 8, 9, 11–13, 15–18, 21–24**

The crude peptides were synthesized and purified similarly to compound **1**, except that each of the unusual amino acids (D-Ser, D-Pro, Phe(p-NO_2), Lys(For)) (4 equiv.) was coupled to the peptide chain using HATU (3.8 equiv.) and DIEA (4.1 equiv.) as coupling reagents. The structure was confirmed by mass spectrometry and data of amino acid analysis. The purity of peptides was estimated by HPLC and TLC.

4.2. Biology

4.2.1. Radioligand binding assay

4.2.1.1. Preparation of cell membranes expressing ORL1 or opioid receptors. This method is a modification of the method of Raynor et al. [20] where CHO cells stably expressing cloned human either μ, δ or κ receptors were harvested by scraping from the culture flasks, centrifuging at $1000 \times g$ for 10 min, resuspending in assay buffer (50 mM tris(hydroxymethyl)aminomethane HCI, pH 7.8, 1.0 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA free acid), 5.0 mM $MgC1_2$, 10 mg L^{-1} leupeptin, 10 mg $^{-1}$ pepstatin A, 200 mg L^{-1} bacitracin, 0.5 mg L^{-1} aprotinin) and centrifuged again. The resulting pellet was resuspended in assay buffer and homogenized with a Polytron homogenizer (Brinkmann, Westbury, NY) for 30 s at a setting of 1. The homogenate was centrifuged at $48{,}000 \times g$ for 10 min at 4 °C and the pellet resuspended at 1 mg protein mL^{-1} of assay buffer and stored at − 80 °C until use.

4.2.1.2. Radioligand binding to ORL1 receptors and to μ, δ, and κ opioid receptors. After dilution in assay buffer and homogenisation as before, membrane proteins (50–100 pg) in 250 pL of assay buffer were added to mixtures containing test compound and radioligand (1.0 nM, 40,000–45,000 dpm) in 250 μL of assay buffer in 96-well deep-well polystyrene titer plates (Beckman) and incubated at room temperature for 60 min ([^{3}H]diprenorphine). The incubation time for [^{3}H]U69,593 was 90 min. Reactions were terminated by vacuum filtration with a Brandel MPXR-96T Harvester through GF/B filters that had been pretreated with a solution of 0.5% polyethylenimine and 0.1% bovine serum albumin for at least 1 h. The filter-bottom plates were washed 4 times with 1.0 mL each of ice-cold 50 mM Tris–HCI, pH 7.8, 30 μL of Microscint-20 (Packard Instrument Company, Meriden, CT) added to each filter, and radioactivity on the filters was determined by scintillation spectrometry in a Packard TopCount.

[^{3}H]Diprenorphine was purchased from Amersham Life Science, Inc. (Arlington Heights, IL) and had a specific activity of 39–45 Ci $mmol^{-1}$. K_D values for diprenorphine binding are 0.33 nM for κ and μ receptors and 0.26 nM for δ receptors. Preliminary experiments were performed to show that no specific binding was lost during the wash of the filters, that binding achieved equilibrium within the incubation time and remained at equilibrium for at least an additional 60 min, and that binding was linear with regard to protein concentration. Non-specific binding, determined in the presence of 10 μM unlabelled naloxone, was less than 10% of total binding.

The data from competition experiments were fit by non-linear regression analysis with the program Prism [21] using the 4 parameter equation for one site competition and subsequently calculating K_i from EC_{50} by the Cheng–Prusoff equation.

4.2.2. MVD assay

Male white mice (24 ± 3 g b.w., MU animal house, Sofia) were killed by cervical dislocation and exsanguinated. The bilateral vasa deferentia were excised and surrounding tissues removed. The preparations (ca. 22 mm long, 2.14 ± 0.11 mg wet tissue weight) were placed horizontally between two ring electrodes (4 mm diameter) located at a distance of 10 and 16 mm from the outlet of 1 mm plastic double-jacketed tissue chambers (37 °C). The tissues were perfused at a constant flow of 0.6 mL min^{-1} by means of a peristaltic pump (Minipuls 2, Gilson) with a medium of the following composition (mM): NaCl 136.9, KCl 2.7, $CaC1_2$ 1.8, $MgSO_4$ 0.6, $NaHCO_3$ 11.9, K_2HPO_4 0.5, glucose 11.5, containing albumin (25 mg mL^{-1}) and Bacitracin (30 mg mL^{-1}), and gassed with 5% CO_2 in O_2 (pH 7.4–7.6). One end of the preparation was tied to a Grass FT 03C force-displacement transducer connected to a Line Recorder TZ 4620 for registration of isometric changes in tension. The preparations were stretched (1.5–2 mN) and allowed to equilibrate for 60 min. The preparations were stimulated electrically for 3 s with trains of rectangular pulses of 50 V, 2 Hz, and 0.6 ms pulse duration every 120 s. The substances were applied at concentrations of 5, 50, 500 and 5000 nM for 20 min with the medium in a non-cumulative manner with 20 min washout periods between each concentration.

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References

[1] J.-C. Meunier, C. Mollereau, L. Toll, C. Suaudeau, C. Moisand, P. Alvinerie, J.-L. Butour, J.-C. Guillemot, P. Ferrara, B. Monsarrat, H. Mazarguil, G. Vassart, M. Parmentier, J. Costentin, Isolation and structure of the endogenous agonist of opioid receptor-like ORL, receptor, Nature 377 (1995) 532–535.

[2] R.K. Reinscheid, H.-P. Nothacker, A. Bourson, A. Ardati, R.A. Henningsen, J.R. Bunzow, D.K. Grandy, H. Langen, F.J. Monsma, Jr., O. Civelli, Orphanin FQ: A novel neuropeptide which is a natural ligand of an opioid-like G protein-coupled receptor, Science 270 (1995) 792–794.

[3] R.K. Reinscheid, A. Ardati, F.J. Monsma, Jr., O. Civelli, Structure activity relationship studies on the novel neuropeptide orphanin FQ, J. Biol. Chem. 271 (24) (1996) 14163–14168.

[4] A.M. Kawasaki, R.J. Knapp, A. Walton, W.S. Wire, T. Zalewska, H.L. Yamamura, E.K. Porreca, T.F. Burks, V.J. Hruby, Synthesis, opioid binding affinities, and potencies of dynorphin A analogues substituted in positions 1, 6, 7, 8 and 10, Int. J. Pept. Protein Res. 42 (1993) 411–419.
[5] C.T. Dooley, R.A. Houghten, Orphanin FQ: receptor binding and analog structure activity relationships in rat brain, Life Sci. 59 (1) (1996) PL 23–29.
[6] A. Turcotte, J.-M. Lalonde, S. St-Pierre, S. Lemaire, Dynorphin-(1–13). I. Structure–function relationships of Ala-containing analogs, Int. J. Pept. Protein Res. 23 (1984) 361–367.
[7] R. Guerrini, G. Calo, A. Rizzi, C. Bianchi, L.H. Lazarus, S. Salvadori, P.A. Temussi, D. Regoli, Address and message sequences for the nociceptin receptor: a structure–activity study of nociceptin-(1–13)-peptide amide, J. Med. Chem. 40 (1997) 1789–1793.
[8] G. Calo, A. Rizzi, G. Bogoni, V. Neugebauer, S. Salvadori, R. Guerrini, C. Bianchi, D. Regal, The mouse vas deferens: a pharmacological preparation sensitive to nociceptin, Eur. J. Pharmacol. 311 (1996) R3–R5.
[9] N. Ling, S. Minick, R. Guillemin, Anino-terminal extension analogs of methionine–enkephalin, Biochem. Biophys. Res. Commun. 85 (2) (1978) 565–570.
[10] R.J. Vavrek, R.-L. Cui, E.J. York, J.M. Stewart, S. Paterson, H.W. Kosterlitz, Lysine substitution increases mu-selectivity of potent di-and tri-peptide enkephalin analogs, Life Sci. 33 (Suppl 1) (1983) 451–454.
[11] J.S. Morley, Structure–activity relationships of enkephalin-like peptides, Ann. Rev. Pharmacol. Toxicol. 20 (1980) 81–110.
[12] A. Turcotte, J.M. Lalonde, S. St.-Pierre, S. Lemaire, Dynorphin(1–13) 1. Structure–function relationships of Ala-containing analogs, Int. J. Pept. Protein Res. 23 (1984) 231–267.
[13] F.-D.T. Lung, J.-P. Meyer, G. Li, B.-S. Lou, D. Stropova, P. Davis, H.L. Yamamura, F. Porreca, V.J. Hruby, Highly κ receptor-selective dynorphin A analogues with modifications in position 3 of dynorphin A(1–11)-NH_2, J. Med. Chem. 38 (1995) 585–586.
[14] F.-D.T. Lung, J.-P. Meyer, B.-S. Lou, L. Xiang, G. Li, P. Davis, L.A. De Leon, H.L. Yamamura, F. Porreca, V.J. Hruby, Effects of modifications of residues in position 3 of dynorphin A(1–11)-NH_2 on κ receptor selectivity and potency, J. Med. Chem. 39 (13) (1996) 2456–2460.
[15] R.E. Chipkin, D.H. Morris, M.G. English, J.D. Rosamond, C.H. Stammer, E.J. York, J.M. Stewart, Potent tetrapeptide enkephalins, Life Sci. 28 (1981) 1517–1522.
[16] P.W. Schiller, B. Eggimann, T. M.-D. Nguyen, Comparative structure-function studies with analogs of dynorphin-(1–13) and [Leu^5]enkephalin, Life Sci. 31 (1982) 1777.
[17] J.-L. Fauchere, P.W. Schiller, Synthesis, characterization and biological properties of five enkephalin-like pentapeptides containing p-nitrophenylalanine, *Helvetica Chim. Acta* 64 (7) (1981) 2090–2094.
[18] H. Yoshino, T. Nakazava, Y. Arakawa, T. Kaneko, Y. Tsuchiya, M. Matsunaga, S. Araki, M. Ikeda, K. Yamatsu, S. Tachibana, Synthesis and structure–activity relationships of dynorphin A(1–8) amide analogues, J. Med. Chem. 33 (1990) 206–212.
[19] J.E. Gairin, R. Gout, J.-C. Meunier, J. Cros, [D-Pro^{10}]-dynorphin(1–11) is a kappa-selective opioid analgesic in mice, J. Pharmacol. Exp. Ther. 245 (3) (1988) 995–1001.
[20] K. Raynor, H. Kong, Y. Chen, K. Yasuda, L. Yu, G.L. Bell, T. Reisine, Pharmacological characterization of the cloned κ-, δ- and μ-opioid receptors, Mol. Pharmacol. 45 (1994) 330–334.
[21] Prism, GraphPad Software, Inc., San Diego (CA).