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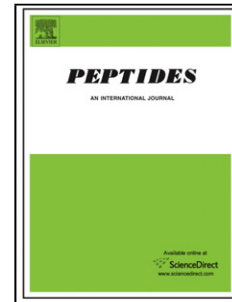

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Biochemical and pharmacological investigation of novel nociceptin/OFQ analogues and N/OFQ-RYYRIK hybrid peptides

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Highlights

- Hybrid peptides are research tools for GPCRs and their interacting complexes
- Nine new ligands were constructed from Dooley peptide RYYRIK and nociceptin fragments
- Domain-like design (message/address) combines the features of the parent peptides
- C-terminal amidation significantly effects the binding and pharmacological character

Abstract

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The endogenous ligand nociceptin (N/OFQ) and a positively charged synthetic peptide RYYRIK are both selective for the nociceptin opioid receptor (NOPr). Despite their structural dissimilarity, N/OFQ and RYYRIK compete for the same binding site of NOP receptor possessing full and partial agonistic character, respectively. In the view of the message-address concept, hybrid peptide constructs were probed for the NOP receptor combining different regions of N/OFQ and RYYRIK related peptide sequences. Nine novel nociceptin- or Ac-RYYRIK-NH_2 peptide variants or hybrid peptides were synthesized and characterized. Peptides P2 and P8 contain fragments of native N/OFQ. The other seven analogues (P1, P3-7, P9) are composed of Ac-RYYRIK-NH_2 fragments and parts of the original nociceptin sequence. The analogues were characterized in receptor binding assays and G-protein activation experiments on rat brain membranes, as well as by electrically stimulated mouse *vas deferens* bioassay. In receptor binding assays ligands P2 , P4 , P6 (K_i 0.37 nM) and P7 showed higher affinity (K_i 0.65 nM, 0.6 nM, 0.37 nM and 0.44 nM, respectively) for NOP receptor than their parent compounds N/OFQ (K_i 2.8 nM) or Ac-RYYRIK-NH_2 (K_i 4.2 nM). In [^{35}S]GTPγS binding experiments P2 and P3 behaved as full agonists. The other variants exhibited partial agonist properties characterized by submaximal stimulatory effects. In mouse *vas deferens* bioassay only P2 showed agonist activity. P4, P5, P6 inhibited the biological activity of N/OFQ more effectively than the NOP receptor selective antagonist JTC-801. In summary, hybrid peptides P4, P5 and P6 proved to be NOP receptor partial agonists even antagonists, while P2 peptide retained the full agonist property.

Keywords: opioid receptor, nociceptin, hybrid peptides, receptor binding,G-protein activation, mouse vas deferens bioassay, bivalent ligands

Introduction

The NOP receptor, belonging to the superfamily of G-protein-coupled receptors, was discovered in 1994, after the cloning of opioid-like orphan receptors [1,2]. It is well known that the N/OFQ-NOP receptor system is widely distributed throughout the nervous system and is involved in the modulation of numerous biological functions, such as pain, depression, reward, learning and memory, food intake, cardiovascular and kidney functions [3]. This multifunctionality of the N/OFQ-NOP receptor system makes NOP receptor a pharmacologically important target. NOP receptor agonists, antagonists and partial agonists may have broad therapeutic potential for treating several disorders. Agonists could be useful

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for treating drug addiction, anxiety, stress, cough and anorexia. Antagonists might be preferable for treating various pain states, depression and Parkinson's disease. Partial agonists that behave as antagonists in the presence of a pure agonist are able to induce water diuresis [3].

The natural endopeptide ligand of NOP receptor is the heptadecapeptide nociceptin or N/OFQ (FGGFTGARKSARKLANQ) [4-6]. The NOP receptor displays high homology with classical opioid receptors (MOP, DOP and KOP receptors), even though it is not able to bind to traditional opioid ligands and is not sensitive to naloxone in binding assays [2,7]. Being an endogenous ligand, N/OFQ binds to the NOP receptor with high affinity and activates it with good efficacy in biochemical experiments. Pharmacological properties of the heptadecapeptide N/OFQ are more complex, because pronociceptive and antinociceptive effects were also described. N/OFQ was originally thought to increase a painful stimulus since intracerebroventricular (i.c.v.) injection of this peptide led to a decrease in tail-flick and hot-plate latency in mice. Further studies suggested that N/OFQ blocks opiate analgesia when administered i.c.v. but potentiates opiate analgesia and has antinociceptive activity when administered intrathecally [7]. The nociceptin ligand shows some homology with endogenous opioid peptides particularly dynorphin A, which is a KOP receptor selective agonist [8,9]. However, N/OFQ shows poor affinity for classical opioid receptors, which is attributed to its *N*-terminal residue Phe (instead of Tyr) [3]. This feature makes nociceptin highly selective for the NOP receptor over other opioid receptors.

Therefore, structure–activity relationship (SAR) studies were performed on N/OFQ to identify the key chemical modifications of its sequence to enhance its potency, metabolic stability, yet modulate its agonist efficacy, which could lead to the development of effective pharmacophores [3,10]. These SAR studies revealed that the main pharmacological features of N/OFQ are retained in the N/OFQ (1-13) fragment and the role of the C-terminal tetrapeptide region (14-17) is negligible.

Besides extensive studies of N/OFQ, screening a hexapeptide combinatorial library led to the identification of several positively hexapeptides with high affinity for the NOP receptor [11], of which Ac-RYYRIK-NH2, Ac-RYYRWK- NH2, Ac-RYYRWR-NH2, Ac-RYYLWR-NH2 and Ac-RYYKWK-NH2 were investigated in further in vitro studies. Although these Dooley's hexapeptides substantially differ in their sequence from nociceptin, they are also highly selective for the NOP receptor over the other opioid receptors behaving as partial agonists, antagonists or full agonist, depending on the tissue preparation or experimental conditions [12,13].

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Comparison of the sequences of N/OFQ and the hexapeptides points to a single common feature, namely all four basic residues of N/OFQ are comprised within a six residue long region (8-13) which, however, is far from the putative message region. Based on the fact that the hexapeptides and N/OFQ show very similar affinity and selectivity profiles, it seems obvious that these hexapeptides contain the message and address information within a significantly shorter fragment.

It is known that the *N*-terminal sequence FGGF of N/OFQ binds deep in the transmembrane binding pocket of the NOP receptor [14], while the positively charged N/OFQ (8-13) binds the negatively charged ECL2 domain of NOP receptor [14], which has a critical role in receptor activation [14,15]. Molecular dynamics simulations suggest that N/OFQ (14-17) does not interact with the NOP receptor [15]. This may be explained by the SAR studies, which show that the shortest active fragment of nociceptin is N/OFQ(1-13) [16,17].

It has been reported that Dooley's hexapeptides such as Ac-RYYRIK-NH_2 competitively inhibited N/OFQ binding to the NOP receptor [18] and therefore their binding sites may overlap [19]. Since Ac-RYYRIK-NH_2 contains basic amino acid residues, similarly to N/OFQ(8-13), it was assumed that this hexapeptide also bound to the acidic ECL2 [20]. However, Kawano et al found that Ac-RYYRIK-NH_2 interacted with the receptor site to which nociceptin(1–7) or -(14–17) binds [19]. Akuzawa et al obtained similar results: they found that Ac-RYYRIK-NH_2 and nociceptin(1-4) shared the NOP receptor binding pocket [21]. Bes and Meunier performed photoaffinity labeling studies using the photo-labile Dooley’s hexapeptide Ac-RYYRWR-NH_2. Their results suggested that, contrary to the previously mentioned two hypotheses, these basic hexapeptides could bind to the TM2 domain of NOP receptor, so N/OFQ and Ac-RYYRYK-NH_2 did not share the binding sites on NOP receptor [20].

In this study nine new nociceptin analogues were constructed. Group 1 of these variants (consisting of 2 peptides denoted by P2 and P8) contained fragments of the natural nociceptin peptide, while Group 2 (7 ligands denoted by P1, P3, P4-7 and P9) included those analogues which were composed of Ac-RYYRIK and parts of the native nociceptin sequence. The hexapeptide motif RYYRIK was present at the *N*-terminus, the middle (P3) or the C-terminus (P9) of these chimeric peptide ligands.

We hypothesized that the hybrid peptides in Group 2, which contain fragments of the N/OFQ sequence and RYYRIK may share the binding sites with nociceptin on the NOP receptor and bind to it with greater affinity than their parent peptides. Furthermore, we

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assumed that those variants in Group 2 which carry Ac-RYYRIK at the *N*-terminus (P1, P4-P7) could competitively inhibit N/OFQ in a highly selective manner.

This paper is devoted to see whether the binding and receptor activation of the NOP receptor by novel synthetic peptide analoguess can be explained by modular interactions by combining the message and address regions of structurally distant peptide ligands. Although an experimental design with all permutations of the supposed domains in NOP (plus RYYRIK and C-terminal amidation added) would supposedly serve more satisfactory answer using a fairly high number of peptides, present paper is addressing only certain key points in the modular building of active N/OFQ analogs. It has been described that the truncated N/OFQ(1-13)-NH_2 showed similar affinity and efficacy as the unmodified N/OFQ, which was explained as a consequence of the *C*-terminal amidation [22]. Therefore, we decided to amidate the *C*-terminus of some analogues (P1-4, P9), to test our hypothesis that this modification improves affinity and potency of these hybrid peptides. The present study aimed to characterize the pharmacological properties (affinity, selectivity and receptor preference) of the nine nociceptin peptide analogues in receptor binding and functional [^{35}S]GTPγS binding assays, as well as in mouse *vas deferens* bioassay. It is expected that hybrid sequences started with the Dooley's motif (P1 and P4-7) will rather be possessing either partial agonist or antagonist properties.

Materials and methods

Radiochemicals

[^{3}H]nociceptin (specific activity: 115.5 Ci/mmol) was purchased from PerkinElmer (Boston, USA). [^{3}H]HS-665 ([23]; s.a: 13.1 Ci/mmol), [^{3}H]DAMGO (s.a: 38.8 Ci/mmol) and [^{3}H]Ile^{5,6}Deltorphin II ([24]; s.a: 19.6 Ci/mmol) were radiolabeled by the Laboratory of Chemical Biology group in BRC (Szeged, Hungary). Guanosine 5'-O-γ-thio-[^{35}S]-triphosphate ([^{35}S]GTPγS; s.a: 1000 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany).

Peptides

Nociceptin(1-17) and Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) were obtained from Bachem Holding AG (Bubendorf, Switzerland). [D-Ala^{2},D -Leu^{5}]enkephalin (DADLE) was purchased from Sigma-Aldrich (Budapest, Hungary). [Ile^{5,6}]-Deltorphin II (IleDelt II) was synthetized in the laboratory of the Chemical Biology group of the Biological Research

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Center (BRC), Szeged, Hungary. The novel peptides were prepared as follows: All solvents and coupling reagents were purchased from VWR (Radnor, PN, USA). Fmoc amino acids and Fmoc-Rink-amide MBHA resin (0.68 mmol/g) were purchased from Chem-Impex (Wood Dale, IL, USA) and IRIS Biotech GmbH (Marktredwitz, DH, Germany). Amino acids were used with orthogonal protecting groups as follows: OtBu for Tyr, Thr, Ser side chains, Pbf protecting group for Arg side chain, Boc protecting group for Lys side chain and Trt for Asn and Gln side chains [21]. The peptides were synthesized by Fmoc-SPPS (standard solid phase peptide synthesis) using TBTU/HOBt/DIPEA for coupling reactions and piperidine 20% solution in DMF for Fmoc group deprotection as previously described [22,23].

Purification of the crude peptides were carried out by RP-HPLC using a Kromasil 100-5C18, 4.6 mm x 250 mm, 5 micron column, at a flow rate of 4.0 mL/min and a linear gradient of H_2O/acetonitrile/0.1% TFA ranging from 5% acetonitrile to 95% acetonitrile in 35 min.

The purity of all final TFA salts was confirmed by analytical RP-HPLC (C18-4.6 mmX250 mm, 5 micron column) at a flow rate of 1 mL/min, using eluent as a gradient of H_2O/acetonitrile 0.1% TFA ranging from 15% acetonitrile to 45% acetonitrile in 12 min and was found to be ≥95%. The identity of final peptides was confirmed by ESI-LRMS. The mass spectrometry (MS) system consisted of an API 150EX, (Applied Biosystems) ion trap mass spectrometer (Foster City, CA) equipped with an electrospray ionization (ESI) source. The capillary temperature was set to 300 °C and the spray voltage to 4.00 kV. The fluid was nebulized using nitrogen (N_2) as the sheath and auxiliary.

Other chemicals

Tris-HCl, $MgCl_2$ x $6H_2O$, NaCl, EGTA and GTP analogue GTPγS were purchased from Sigma-Aldrich (Budapest, Hungary). SB-612 and JTC-80 were purchased from Tocris Bioscience (Bristol, UK). Naloxone was kindly provided by the company Endo Laboratories DuPont de Nemours (Wilmington, DE, USA). The UltimaGold^{TM} MV aqueous scintillation cocktail was purchased from PerkinElmer (Boston, USA).

Animals

In *in vitro* receptor binding experiments we applied inbred Wistar rats (250-300 g body weight) which were housed in the local animal house of BRC (Szeged, Hungary), and male guinea pigs (~700 g body weight, LAL/HA/BR strain) which originated from LAB-ÁLL Bt. (Budapest, Hungary). In *mouse vas deferens* bioassay we used NMRI mice (35-45 g). Mice were purchased from Toxicoop (Budapest, Hungary) and were housed in the local animal

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house of the Department of Pharmacology and Pharmacotherapy of the Semmelweis University (Budapest, Hungary).

All animals were kept in groups of five in a temperature controlled room (21-24 ºC) under a 12:12 light and dark cycle, allowed free access to tap water and standard rodent food until the time of sacrifice. The animals were handled humanely, in complete accordance with the European directive 2010/63/EU on the protection of animals used for scientific purposes and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). Both the number of animals and their suffering were minimized throughout our experiments.

Membrane preparation

Preparation of rat brain membranes

Throughout the *in vitro* receptor binding experiments we applied rat and guinea pig brain membranes. The membrane fractions were prepared according to the method described previously [24]. Briefly, the animals were decapitated and the brains were quickly removed. The brains without the cerebellum were homogenized in ice-cold 50 mM Tris–HCl buffer (pH 7.4) using a Teflon-glass homogenizer. The filtered homogenates were centrifuged at 40,000 × g for 20 min at 4 °C. Afterwards the pellet was resuspended in 50 mM Tris-HCl (pH 7.4) buffer and incubated at 37 °C for 30 min in a shaking water-bath. The centrifugation step was repeated after the incubation. The final pellet was suspended in 50 mM Tris-HCl (pH 7.4) buffer containing 0.32 M sucrose and stored at -85 °C until further use.

Radioligand Binding Competition Experiments

Aliquots of frozen rat and guinea pig brain membrane homogenates (40,000 x g, 20 min, 4 °C) were centrifuged and washed to remove sucrose. The pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4) with up to 0.3-0.5 mg/ml of protein. Membranes were incubated with 1 nM of the radioligands and increasing concentrations (10^{-10}-10^{-5} M) of the various unlabeled peptides to be tested. Incubation conditions depended on the radioligands: for [^{3}H]DAMGO and [^{3}H]Ile^{5,6}Deltorphin II 35 °C for 45 min, for [^{3}H]HS-665 30 °C for 45 min, and for [^{3}H]nociceptin 30 °C for 30 min. Guinea pig brain was used in those experiments in which the tested ligands were investigated for KOP receptor, because it has significantly more KOP receptors than rat brain. Total binding was measured in the presence of the given radioligands, while non-specific binding was determined in the presence of 10 μM of unlabeled naloxone, naltrindole, U-69,593 and nociceptin, respectively. After the incubation, the reaction was terminated by filtrating the samples through Whatman GF/C ([^{3}H]DAMGO,

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[^{3}H]Ile^{5,6}Deltorphin II or GF/B ([^{3}H]U-69593 and [^{3}H]nociceptin) glass fibre filters (GE Healthcare, Little Chalfont, UK) under vacuum (Brandel M24R Cell Harvester; Brandel Harvesters, Gaithersburg, MD). The filters were washed three times with 5 ml ice-cold 50 mM Tris-HCl (pH 7.4) and then with UltimaGold MV aqueous scintillation cocktail (Perkin Elmer, Waltham, MA) to detect the radioactivity with a Packard Tricarb 2300TR LSC spectrometer. The competition binding assays were performed in duplicates and repeated at least three times.

[^{35}S]GTPγS Binding Experiments

The functional [^{35}S]GTPγS binding assays were performed as described previously [25], with slight modifications. Rat and guinea pig brain membrane fractions (~10 μg of protein/sample) were incubated at 30 °C for 60 min in Tris-EGTA buffer. The buffer contained 50 mM Tris-HCl, 1 mM EGTA, 3 mM MgCl2, 100 mM NaCl, 30 μM GDP, (pH 7.4), as well as 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM) and increasing concentrations (10^{-10} – 10^{-5} M) of the tested nociceptin analogues and hybrid peptides. To investigate the NOP receptor selectivity 10 μM NOP receptor specific antagonist SB-612,111 was used. The final volume was 1 ml. Afterwards total binding (T) in the absence of the tested compounds was determined, and non-specific binding (NS) in the presence of 10 μM unlabeled GTPγS to calculate basal activity.

The reaction was terminated after incubation and bound and unbound [^{35}S]GTPγS were separated. The radioactivity of the filters was measured in the same way as described before.

Isolated tissue assay

Mouse *vas deferens* Bioassay

Mouse *vas deferens* (NMRI mice, 35-45 g) was prepared as described before [26]. After the preparation *vas deferens* were suspended in 5 ml organ baths containing Mg^{2+} free Krebs buffer (118.1 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 11 mM glucose, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$) and aerated with carbogen (O_2:CO_2=95:5) at 31 °C. The tissues were mounted between two electrodes under an initial tension of 0.1g. The stimulation parameters were as follows: field stimulation, pairs (100 ms pulse distance) of rectangular impulses (1 ms pulse width, 9V/cm i.e. supramaximal intensity) were repeated by 10 s. The muscle contractions were monitored by computer, using a data recording and the analysis system LabChart 5 (ADInstruments Pty LTD, Australia).

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Before adding the first dose of agonists, 30–40 min equilibration was performed on the tissues under stimulation. Then the cumulative concentration-response curves of to nociceptin analogues, hybrid peptides , nociceptin(1-17), or the control compounds were constructed.

This method was suitable for separating peptides with agonist activity from peptides with antagonist activity. To determine the dissociation constants (Ke) of the antagonists,

the single-dose method was used [27].

Data Analysis and Terminology

All the experiments were repeated at least 3 times and the data were expressed as means ± standard error of mean (S.E.M.) in logarithmic form. The radioligand competition binding experiments were performed in duplicates, while the $[^{35}S]GTP\gamma S$ binding assays were carried out in triplicates. The experimental data were analyzed and points were fitted with GraphPad Prism 6.0 (GraphPad Prism Software Inc., San Diego, CA, USA, www.graphpad.com) using non-linear regression. "One-site competition" was applied in the radioligand competition binding assays to determine unlabelled ligand affinity (IC_{50}). In the $[^{35}S]GTP\gamma S$ binding assays the 'Sigmoid dose-response' equation was used to determine ligand potency (EC_{50}) and maximum G-protein efficacy (E_{max}). The curve fitting program outputs the logarithm of the standard errors of mean (S.E.M.) for IC_{50} and EC_{50} values, due to the data representation. The equilibrium inhibition constant (K_i) was calculated according to the Cheng-Prusoff equation: $K_i= IC_{50}/(1+[L]/K_d)$ [28] where IC_{50} is defined as the concentration of the competitor which produces 50% displacement, and [L] designates the concentration of the labelled ligand. Basal activity was determined in $[^{35}S]GTP\gamma S$ binding assays in the absence of receptor ligands, and was settled at 100%. The data were normalized to the basal activity. Agonist potencies were expressed as pEC_{50}. The E_{max} is the maximal effect that an agonist can trigger in a given tissue or preparation. In the $[^{35}S]GTP\gamma S$ binding assay E_{max} of agonists represents the maximal stimulation over the basal and it is expressed in %.

After the analysis of experimental data from the MVD bioassay, EC_{50} were determined from individual logarithmic concentration-response curves. The equilibrium dissociation constant of antagonists (K_e) was calculated using the single-dose method [27]. K_e values were calculated with the following formula: K_e = concentration of antagonist/[dose ratio − 1] [29]. Unpaired t-test with two-tailed p-value was used to see the differences between the two data sets, while for three or more data sets one-way ANOVA with Tukey's post hoc test was

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performed to determine statistical significance. Significance was accepted at the $p < 0.05$ level.

Results

Receptor binding assays

The sequences of the newly synthesized and characterized nociceptin analogues and nociceptin-RYYRIK hybrid peptides are summarized in **Table 1**, sorted into two groups: Group 1 consists of 2 ligands denoted as P2 and P8, which do not contain the RYYRIK motif, while Group 2 is comprised of 7 peptides denoted as P1, P3-7 and P9**.**

In order to determine the NOP receptor binding affinity of the novel peptides radioligand competition binding assays were carried out with [^{3}H]nociceptin on rat brain membranes. For control their parent compounds (nociceptin and Ac-RYYRIK-NH_2) were also examined.

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Table 1.

List of fused hybrid peptides and their parent compounds with abbreviated names and sequences.

Name/code	Length	Sequence
Parent peptides		
Nociceptin, N/OFQ	**17**	H-FGGFTGARKSARKLANQ-OH
Dooley's peptide	**6**	Ac-RYYRIK-NH_2
Group 1 (without RYYRIK)		
P2	**17**	H-FGGFTSARKGARKLANQ-NH_2
P8	**11**	H-FGGFGGGFGGF-NH_2
Group 2 (containing RYYRIK)		
P1	**14**	Ac-RYYRIKGARKLANQ-NH_2
P3	**18**	H-FGGFRYYRIKSARKLANQ-NH_2
P4	**14**	Ac-RYYRIKSARKLANQ-NH_2
P5	**14**	Ac-RYYRIKSARKLANQ-OH
P6	**18**	Ac-RYYRIKGARKSARKLANQ-OH
P7	**14**	Ac-RYYRIKGARKSARK-OH
P9	**13**	H-FGGFGGGRYYRIK-NH_2

As shown in **Fig. 1AB** and **Table 2**, peptides P1-P7 competed more effectively for NOP receptor than P8 or P9, which showed negligible affinity towards NOP receptor.

Surprisingly, P3 also showed low affinity in spite of FGGF being present at its *N*-terminal. On the other hand, P1, P2, P4, P6 and P7 displayed higher affinity to NOP receptor compared to the parent compounds. Although P2 differs from nociceptin in that it contains Ser^6 instead of Gly^6, and Gly^{10} instead of Ser^{10}, we observed a remarkable affinity (K_i=0.65) against [^{3}H]nociceptin.

The chimeric peptides possessing Ac-RYYRIK N-terminal were tested for KOP, MOP and DOP to reveal selectivity within the opioid receptor family. The results showed that none of the nociceptin-RYYRIK hybrid peptides displayed DOP receptor affinity (data not shown). However, [^{3}H]DAMGO was almost fully displaced from its receptor by P4 in higher concentrations. P1 and P7 showed relatively high affinity in the MOP receptor binding (K_i=13 and 209 nM) (data not shown).

In competition binding assays performed with [^{3}H]HS665, P4, P6 and P7 compounds fully displaced [^{3}H]HS665 binding, however the inhibition was in the micromolar concentration range (data not shown).

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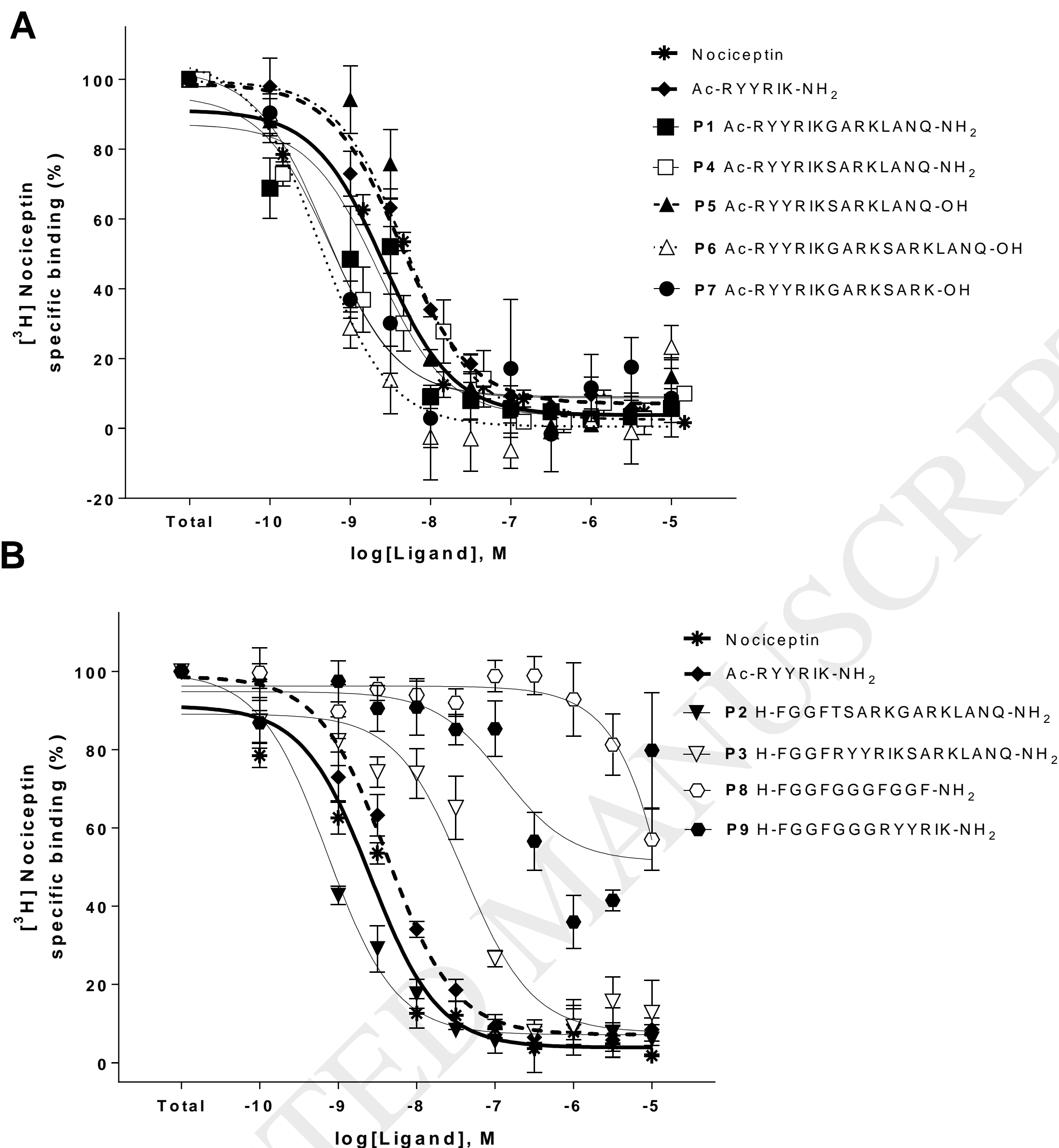

Figure 1. NOP receptor binding affinity of nociceptin hybrid peptides (**A** and **B**) compared to nociceptin and Ac-RYYRIK-NH_2 in [^{3}H]nociceptin competition binding assays in rat brain membrane homogenates. Figures represent the specific binding of [^{3}H]nociceptin in percentage in the presence of increasing concentrations (10^{-10}–10^{-5} M) of the indicated unlabeled ligands. The level of total specific binding was defined as 100% and points represent means ± S.E.M. for at least three experiments performed in duplicates.

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Table 2.

Competition binding data of nociceptin hybrid peptides and their parent compounds in brain membrane homogenates. The $logIC_{50}$ values were calculated according to the competition binding curves (see Supplementary Figures 1 and 2).

		Affinity, pIC50 ± S.E.M. (K_i, nM)		
Compounds		**[^{3}H]HS665 (KOP receptor)**	**[^{3}H]DAMGO (MOP receptor)**	**[^{3}H]nociceptin (NOP receptor)**
Parent compounds				
Nociceptin		9.34±2.9 (0.4)	8.26±0.33 (4.79)	8.5±0.1 (2.8)
Ac-RYYRIK-NH_2		6.22±1.1 (529)	7.3±3.2 (24.6)	8.4±2.5 (4.2)
Fused hybrid peptides				
P1	Ac-RYYRIKGARKLANQ-NH_2	5.8±0.5 (1482)	7.6±2.2 (26)	8.7±0.2 (2)
P2	H-FGGFTSARKGARKLANQ-NH_2	n.d.	n.d.	9.1±0.1 (0.65)
P3	H-FGGFRYYRIKSARKLANQ-NH_2	n.d.	n.d.	7.4±0.12 (36)
P4	Ac-RYYRIKSARKLANQ-NH_2	5.6±0.2 (2510)	5.3±0.4 (4549)	9.3±0.1 (0.6)
P5	Ac-RYYRIKSARKLANQ-OH	5±0.7 (9699)	5.6±3.2 (2432)	8.3±0.1 (4.44)
P6	Ac-RYYRIKGARKSARKLANQ-OH	5.5±0.2 (3202)	4.9±0.7 (12120)	9.4±0.2 (0.37)
P7	Ac-RYYRIKGARKSARK-OH	5.4±0.3 (3998)	6.4±0.4 (422)	9.3±0.2 (0.44)
P8	H-FGGFGGGFGGF-NH_2	n.d.	n.d.	4.2±2.2 (6192)
P9	H-FGGFGGGRYYRIK-NH_2	n.d.	n.d.	6.9±0.3 (112)

n.d.: not determined

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[^{35}S]GTPγS binding experiments

[^{35}S]GTPγS binding experiments were performed on rat brain membranes to determine the potency (pEC_{50}) and efficacy (E_{max}) of the nociceptin analogues and nociceptin-RYYRIK hybrid peptides. The test compounds were compared with their parent compounds, nociceptin, and Ac-RYYRIK-NH_2.

As it can be seen in **Table 3** all the peptides moderately stimulated the monitored G-protein except for P2 and P3. While P1, P4-P9 indicated partial agonist properties, P2 and P3 exhibited full agonist activity similar to nociceptin (E_{max}: 157%, 152% and 148% for P2, P3 and nociceptin, respectively).

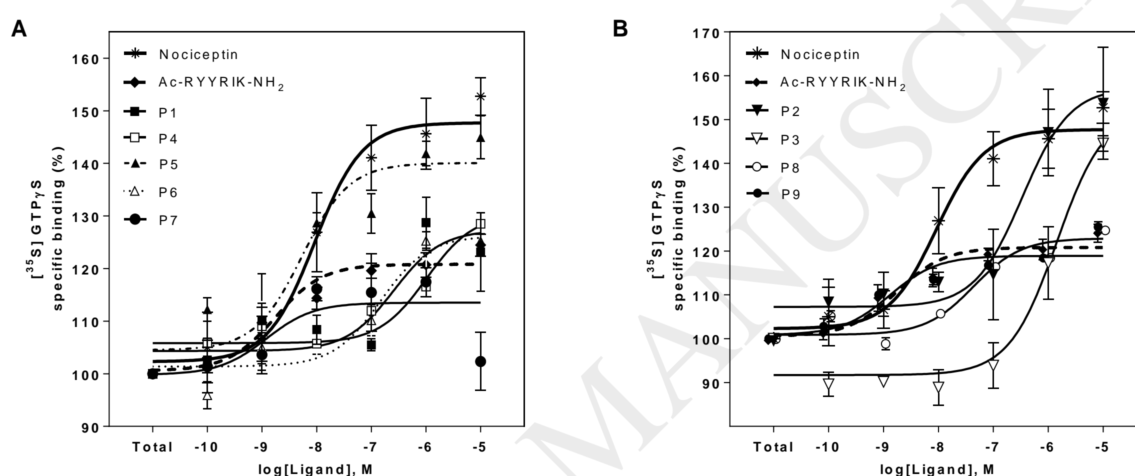

Figure 2. [^{35}S]GTPγS binding experiments in rat brain membranes. Panel A: stimulation of G-protein activation by nociceptin analogues. Panel B: nociceptin-RYYRIK hybrid peptides induced activation of G-proteins. Points represent means ± S.E.M. for at least three repeated experiments performed in triplicate.

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Table 3. The stimulation of G-protein by the nociceptin hybrid peptides and their parent compounds in [^{35}S]GTPγS binding assays using rat brain membrane homogenates. The efficacy (E_{max}) and ligand potency (pEC_{50}) values were calculated by analyzing dose-response binding curves.

	Peptides	Rat brain membranes	
		Potency	**Efficacy**
		pEC_{50} ± S.E.M. (EC_{50}, nM)	% E_{max}, ± S.E.M.
	Parent compounds		
	Nociceptin	8.0±0.2 (9.2)	148±3.6
	Ac-RYYRIK-NH_2	8.76±0.2 (2)	121±1.2
	Fused hybrid peptides		
P1	Ac-RYYRIKGARKLANQ-NH_2	6.6±0.5 (258)	127±4.6
P2	H-FGGFTSARKGARKLANQ-NH_2	6.5±0.35 (338)	157±0.34
P3	H-FGGFRYYRIKSARKLANQ-NH_2	5.85±0.28 (1430)	152±8.2
P4	Ac-RYYRIKSARKLANQ-NH_2	6.9±0.41 (119)	119±3.65
P5	Ac-RYYRIKSARKLANQ-OH	8.3±0.3 (5.4)	140±2.6
P6	Ac-RYYRIKGARKSARKLANQ-OH	7.5±0.6 (147)	126±2.33
P7	Ac-RYYRIKGARKSARK-OH	8.93±0.51 (1.2)	114±2.5
P8	H-FGGFGGGFGGF-NH_2	7.4±1.2 (44)	123±1.02
P9	H-FGGFGGGRYYRIK-NH_2	8.9±1.3 (1.3)	119±1.01

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To reverse or inhibit the G-protein stimulatory effect of the test compounds, we applied NOP receptor specific antagonist SB612.111 in 10 µM concentrations (**Fig.3**). The stimulating effects of P2 and P3 were significantly antagonized by SB612.111, which confirms that NOP receptor is involved in mediating the effects of these peptides.

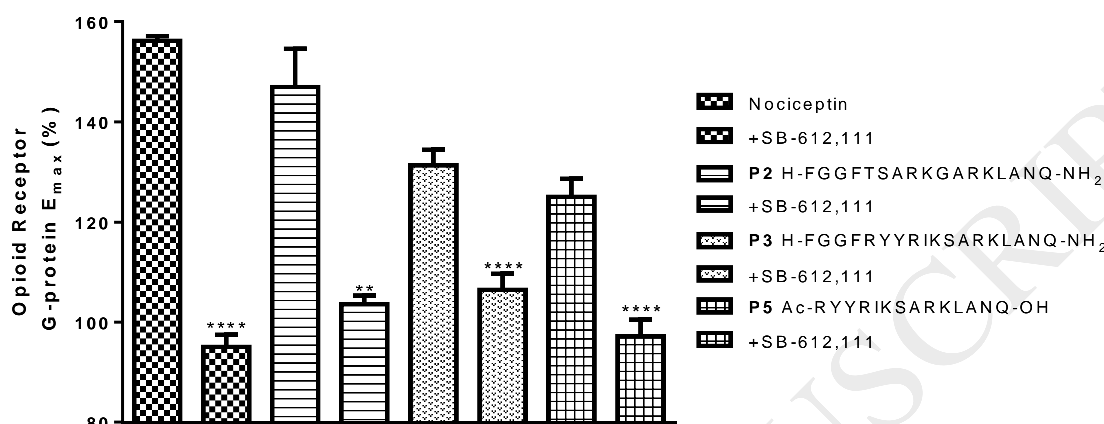

Figure 3. Effect of a nociceptin receptor specific antagonist ligand (SB-612,111) on the G-protein activation by the nociceptin hybrid peptides. Columns shown represent mean values ± S.E.M. SB-612,111 was used in 10 µM concentration. **p < 0.01 and ****p < 0.0001 vs. control group (unpaired t-test with Welch's correction, two-tailed p-value).

Mouse *vas deferens* bioassay

The aim of the mouse vas deferens bioassay was the clarification the agonist or antagonist activity of the nociceptin analogues and nociceptin-RYYRIK hybrid peptides. As a first step we set out their ability of twitch response inhibition to electrical field stimulation without a NOP receptor antagonist to determine their agonistic activity. As detailed in **Table 4**, the tested peptides showed slight inhibition only at high concentrations (i.e., 10 µM) even in the case of P2. Although P2 displayed high affinity (K_i=0.65) for NOP receptor in radioligand competition binding assays and high efficacy (157%) in $[^{35}S]$GTPγS binding experiments, we only observed weak affinity for P2 (426 nM) in comparison with nociceptin (62.4 nM) in mouse *vas deferens* bioassay. After characterizing the agonist activity of the tested peptides, we investigated their antagonist activity in the presence of nociceptin. The affinities of antagonists (K_e) are summarized in **Table 4**. P5 (K_e=136 nM, n=9), P6 (K_e=166 nM, n=6) and P4 (K_e=258 nM, n=5) could antagonize the effect of nociceptin more effectively than the control antagonist JTC-801 (K_e=480 nM, n=11), which displayed a negligible partial agonist activity in high concentration (10 µM) similar to P4, P5, P6 and P7.

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Table 4. Effects of the nociceptin hybrid peptides and reference compounds on the electrically stimulated mouse *vas deferens*.

	Ligands	EC_{50} (nM) ± S.E.M.	K_e values for antagonists ± S.E.M. (nM)
	Control compounds		
	Nociceptin	62±4	n.e.
	JTC-801	>100000	480±72
	Fused hybrid peptides		
P1	Ac-RYYRIKGARKLANQ-NH_2	>10000	1943±341
P2	H-FGGFTSARKGARKLANQ-NH_2	426±76	n.e.
P3	H-FGGFRYYRIKSARKLANQ-NH_2	>1000000	n.e.
P4	Ac-RYYRIKSARKLANQ-NH_2	>100000	258±61
P5	Ac-RYYRIKSARKLANQ-OH	>1000000	136±26
P6	Ac-RYYRIKGARKSARKLANQ-OH	>100000	166±31
P7	Ac-RYYRIKGARKSARK-OH	>1000000	507±79
P8	H-FGGFGGGFGGF-NH_2	>1000000	n.e.
P9	H-FGGFGGGRYYRIK-NH_2	>1000000	n.e.

n.e.: no effect

Discussion

The single natural ligand of the G-protein coupled NOP receptor, nociceptin (N/OFQ, FGGFTGARKSARKLANQ) was identified in 1995 via reverse pharmacology [3,4]. N/OFQ together with its NOP receptor forms the N/OFQ-NOP system, which has been intensively studied over the past 20 years. The investigations have revealed that both NOP receptor agonists and antagonists have significant therapeutic potential. Structure–activity relationship (SAR) studies were performed to identify those amino acids and/or sequences in the N/OFQ sequence that are essential for binding to and activation of NOP receptor. Furthermore, such chemical modifications were investigated that could increase potency, metabolic stability and agonist efficacy, or alter the agonist and antagonist properties.

In this study 9 novel nociceptin variants were synthesized and characterized. Group 1 of these variants comprises peptides P2 and P8, which contain fragments of the natural nociceptin peptide. Group 2 includes those analogues (P1, P3, P4, P5, P6, P7, P9) which are

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composed of the Ac-RYYRIK-NH_2 hexapeptide and parts of the original nociceptin sequence. RYYRIK could be present at the beginning (*N*-terminal), the middle or the end (*C*-terminal) of these synthetic peptides. We chose Ac-RYYRIK-NH_2 because it acted as an antagonist in the presence of nociceptin and inhibited its biological activity.

Initially it was reported that transmembrane helix 2 of the NOP receptor takes part in the recognition of Ac-RYYRIK-NH_2, but other molecular modelling and docking studies found that Ac-RYYRIK-NH_2 interacts with those sits of NOP receptor such as N/OFQ, which implies that the binding sites of N/OFQ and RYYRIK may overlap. One of the implications of this result is that hybrid peptides from the combination of RYYRIK and parts of nociceptin may bind to NOP receptor with greater affinity than nociceptin itself.

The 9 peptides were first studied in receptor binding assays. P8 (H-FGGFGGGFGGF-NH_2) and P2 (H-FGGFTSARKGARKLANQ-NH_2) belonging to Group 1 had different affinities for the NOP receptor. While P8 was practically unable to displace [^{3}H]nociceptin, the K_i value of P2 was lower than that of nociceptin. By synthesizing P8 our aim was to investigate whether we could create a high affinity peptide resembling little bit to biphalin [30]. However, the absence of the address domain resulted in a loss of binding ability probably because of the lack of some basic residues and other residues essential for binding. The sequences of P2 and N/OFQ are different, because N/OFQ contains the <u>G</u>ARK<u>S</u>ARK central motif, which is replaced with the <u>S</u>ARK<u>G</u>ARK sequence in P2, so the positions of the apolar Gly and the polar Ser have changed. A further difference is that P2 bears an amidated C-terminus. In terms of binding affinity at the NOP receptor P2 proved to be the best peptide analogue among the compounds studied. Similarly to the endogenous N/OFQ sequence, P2 exhibited a full agonist profile (E_{max} 157%) although with lower potency in the [^{35}S]GTPγS binding experiments. Beside N/OFQ, P2 was the other peptide carrying pure agonist activity in the mouse *vas deferens* bioassay. Taken together, despite the three modifications, the biochemical and pharmacological properties of P2 and N/OFQ remained close to each other.

The variants belonging to Group 2 (P1, P4, P5, P6 and P7) displaced [^{3}H]nociceptin with high affinity. P4, P6 and P7 showed lower K_i values than even the parent peptides, indicating increased affinities. The cause of these high affinity values may well be that these peptides combine the favorable properties of RYYRIK and N/OFQ. Kawano et *al.* found that RYYRIK is likely to interact with the site of NOP receptor where nociceptin(1-7) and nociceptin(14-17) are involved [19]. Experimental results show that RYYRIK binds to the N/OFQ binding site with greater affinity than the original FGGF message domain, for which

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the Ac-RYY residues are primarily responsible. P4, P6 and P7 also contain the nociceptin address domain. Thus, by fusion of RYYRIK and N/OFQ, we could combine their beneficial properties that may have contributed to the increase of affinity.

It is worth noting that although the amino acid sequences of P4 and P5 are identical, P4 had a 7 times greater affinity (K_i =0.6 nM) than P5 (K_i =4 nM) towards the NOP receptor. This is explained by the fact that the C-terminus of P4 was amidated while that of P5 was not. It has already been shown that the truncated and amidated nociceptin(1-13)-NH_2 exhibits a binding affinity and biological activity similar to the unmodified N/OFQ, in contrast with the deamidated truncated nociceptin(1-13), which showed poorer affinity and activity [22].

It was interesting to see that P1, which contains Gly^7 instead of Ser^7, displayed 30% lower K_i value than P4 toward the NOP receptor. We assume that the apolar Gly at position 7 in the P1 ligand caused the reduction in affinity.

Although it is well known that N/OFQ and RYYRIK bind selectively to the NOP receptor, we aimed to investigate whether hybrid peptides made of highly charged RYYRIK fused with portions of N/OFQ could retain their NOP receptor selectivity, since the sequence of nociceptin peptide exhibits some similarity with the endogenous KOP receptor selective dynorphin A, e.g. message domain, and many charged amino acids in the address domain.

The results showed that the hybrid compounds had a weaker affinity for the KOP receptor than the parent compounds, so it could be excluded that hybridization of the two peptides could increase KOP receptor selectivity at the expense of NOP receptor selectivity.

In $[^{35}S]GTP\gamma S$ assays, P8 acted as a partial agonist, while the E_{max} value of P2 was higher than that of N/OFQ. It should be noted that while P2 had an EC_{50} of 338 nM (**Table 3**), the EC_{50} value of N/OFQ is 1.9 nM, i.e. N/OFQ is over 36x more potent as P2. Of note, drugs of high efficacy have advantage in therapy, because they are able to activate NOP receptor in pathological conditions where NOP receptor reserve is low.

It has been demonstrated in case of N/OFQ that substituting the apolar - neutral Gly^6 to Ala may slightly reduce the potency [10]. In the light of the fact that the potency has greatly decreased in our case, it is assumed that the replacement of the apolar amino acid with a more polar Ser^6 was responsible for the large decrease.

P4, P6 and P7 showed partial agonist activity while P5 was a full agonist in the G-protein assay. Of the five variants, the best EC_{50} was shown by P5 (1.2 nM) and P7 (5.4 nM). The E_{max} value of peptide P4 was 131 ± 0.42, while that of P5 was 140 ± 2.6. This contradicts our expectations, according to which P4 (which was amidated at the *C*-terminal end) was

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expected to show full agonist activity. However, P4 showed lower potency and lower E_{max} (1028 nM) values as expected. Although P9 produced rather weak efficacy (E_{max} = 119 ± 1.01), its potency was still strong. Conversely, P3 produced high E_{max} value with substantially weaker potency (1430 nM). We assume that this weak effect could have arisen from the insertion of the RYYRIK hexapeptide into the nociceptin sequence. The role of the positively charged segment was addressed by peptides P3 and P9: In P9 the N-terminal FGGF (essential in N/OFQ) was connected to RYYRIK by the GGG spacer positioning the first Arg residue to the same place where N/OFQ holds the first Arg residue (Arg^8). At a time the minimal active length of 13 (in N/OFQ) was kept. The very week affinity of the resulting peptide, however, points to the importance of sequence specific interactions rather than to the position of the charged segment. P3 showed somewhat higher affinity, presumably due to the presence of the natural address region (in a more or less the correct distance from the N-terminal) and the Arg^8 residue essential in N/OFQ.

Variants that showed full agonist activity (P2, P3, P5) in the G-protein assay, were also tested for NOP receptor selectivity. To achieve this, an effective NOP receptor selective antagonist, SB612, was used. The Fig. 3 clearly shows that the E_{max} of P2, P3 and P5 were decreased in the presence of the antagonist. This demonstrates that our hybrid peptide ligands affected NOP receptor in a selective way.

Following in vitro biochemical studies, the pharmacological effects of the peptide variants were also investigated in mouse *vas deferens* bioassay. First, the ligands were tested alone to determine their agonist potency and efficacy. The studied ligands did not exhibit agonist activity, except for P2, in the case of which the EC_{50} was 426 ± 76 nM, while it was over 10 μM for all other peptides.

This result is surprising because the ligands proved to be promising in both binding and [^{35}S]GTPγS tests based on the K_i, EC_{50} and E_{max} values. Apart from this, the peptides are considered worthy of further investigations. Furthermore, it is important to note that, although P3 was also expected to achieve better agonist activity based on its E_{max} value (which was better than that of N/OFQ), despite our expectations, this ligand was inactive in the bioassay.

Only those ligands were expected to have antagonistic activity that contained RYYRIK at their *N*-terminal because RYYRIK itself acted as antagonist in previous [^{35}S]GTPγS and mouse *vas deferens* experiments. Our results confirmed these expectations, as partial agonist ligands that did not contain RYYRIK, or did contain RYYRIK but not at their *N*-terminus (P9), proved to be inactive. P8 and P9 were found to be partial agonists in the [^{35}S]GTPγS

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binding experiments, but they were completely inactive in the mouse vas deferens bioassay. In the radioligand binding studies only P9 exhibited a relatively high affinity, while P8 bearing two FGGF tandem 'message' motifs was practically inactive (Ki > 6 μM). One of the longest hybrid octadecapeptide variants P3 also showed high affinity in competition binding assays and high efficacy in [^{35}S]GTPγS binding tests, but it was fully inactive in mouse *vas deferens* bioassay. These findings suggest that the use of a diversified experimental approach is better in characterizing novel synthetic analogues with previously unknown properties.

Summarizing the results, those ligands have been shown to be the most potent antagonists that carry RYYRIK on the *N*-terminus. These results suggest that the binding sites of RYYRIK and nociceptin on NOP receptor may overlap. However, it cannot be ruled out that RYYRIK binds to the NOP receptor at the TM2 domain. For P2 it can be established that, although it bound to NOP receptor with greater affinity than N/OFQ, and its E_{max} value was also greater than that of nociceptin, its EC_{50} was lower in both GTP and MVD tests than the EC_{50} of nociceptin (36 times and 7 times, respectively). It is worth mentioning that, despite the fact that P5 performed worse than P4 in the binding tests, the receptor activity test showed full agonist activity, and its potency was 190 times higher than that of P4. The mouse *vas deferens* bioassay also showed that P5 acts as a more effective antagonist than P4. This contradicts our hypothesis that the amidation of the *C*-terminus increases the affinity and biological activity of N/OFQ peptide analogues. The present data demonstrated that only one of our experimentally studied peptides, P2 retained the full agonist property, while those hybrids which contain the Dooley's sequence at their N-terminal (P1 and P4-7) exhibited partial agonism with significant antagonist activity. Ultimately, it would be useful to further investigate these peptides in *in vivo* experiments as well.

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