Published in final edited form as:

J Med Chem. 2009 July 9; 52(13): 4068–4071. doi:10.1021/jm900604g.

Further studies at neuropeptide S position 5: discovery of novel neuropeptide S receptor antagonists

Remo Guerrini^{§,*}, Valeria Camarda[#], Claudio Trapella[§], Girolamo Calo^{'#}, Anna Rizzi[#], Chiara Ruzza[#], Stella Fiorini[§], Erika Marzola[§], Rainer K. Reinscheid[¶], Domenico Regoli[#], and Severo Salvadori[§]

[§]Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy

[#]Department of Experimental and Clinical Medicine, Section of Pharmacology and Neuroscience center, and National Institute of Neuroscience, University of Ferrara, via Fossato di Mortara 19, 44100 Ferrara, Italy

[¶]Department of Pharmaceutical Sciences, University of California Irvine, 360 Med Surge 2, Irvine, CA 92697, USA

Abstract

Neuropeptide S (NPS) regulates various biological functions by activating the NPS receptor (NPSR). Previous studies demonstrated that the substitution of Gly⁵ with D-amino acids generates NPSR antagonists. Eleven [D-Xaa⁵]NPS derivatives were synthesized and pharmacologically tested measuring [Ca²⁺]_i in HEK293_{mNPSR} cells. The present results confirmed that the [D-Xaa⁵] substitution promotes antagonist activity with potency inversely related to the side chain size and allowed to identify the novel potent NPSR peptide antagonist [tBu-D-Gly⁵]NPS.

Introduction

Neuropeptide S (NPS, human sequence SFRNGVGTGMKKTSFQRAK) is the endogenous ligand of the 7TM receptor NPSR.¹ Via selective NPSR activation, NPS regulates several biological functions including wakefulness^{1, 2}, stress and anxiety^{1–5}, locomotor activity^{1–3}, food intake^{6–8}, memory processes^{5, 9}, and drug abuse^{10–13}. To deeply investigate these NPS sensitive biological functions and to identify the therapeutic potential of drugs interacting with the NPSR, potent and selective ligands are required. With the aim to identify NPSR ligands structure activity relationship (SAR) studies were performed on the NPS sequence that allowed to demonstrate the crucial importance of the sequence Phe²-Arg³-Asn⁴ for receptor binding and activation^{14–16} and that of the sequence Gly⁵-Val⁶-Gly⁷ for shaping NPS into the biologically active conformation^{14, 16, 17}. Few SAR studies were then performed on Phe² (ref¹⁸), Arg³ and Asn⁴ (ref¹⁹). More recently, a SAR study focused on Gly⁵ was carried out.²⁰ This investigation demonstrated that the introduction in NPS position 5 of a chiral center with relative configuration D, produces important changes in peptide potency and, particularly, in its efficacy. In fact, the replacement of Gly⁵ with D-Leu or D-Cys generated NPSR partial agonists while that with D-Met or D-Val produced pure and fairly potent NPSR antagonists.

*To whom Correspondence should be addressed. Remo Guerrini, Phone: +39-0532-455-988; Fax: +39-0532-455953. E-mail: r.guerrini@unife.it.

Supporting Information Available: a table reporting the retention time determined by analytical HPLC analyses using two different chromatographic systems and the calculated and found molecular weight of the compounds. This material is available free of charge via the internet at <http://pubs.acs.org>

The NPSR antagonistic properties of [D-Val⁵]NPS were confirmed in vivo in the mouse locomotor activity assay, where the peptide at 10 nmol, blocked the stimulatory effect elicited by the supraspinal administration of 0.1 nmol NPS.²⁰ These findings prompted us to further investigate position 5 with the aim of understanding the chemical requirements of the D-amino acid side chain that are instrumental for generating NPSR antagonism.

Results and Discussion

Eleven novel peptides (Table 1) were synthesized in good yield and with a purity grade not less than 95% following procedures previously described.¹⁸ NPS, [D-Val⁵]NPS (used as reference NPSR antagonist), and the novel peptides were pharmacologically evaluated in a calcium mobilization assay using HEK293 cells stably expressing the mouse NPSR (HEK293_{mNPSR}). The protocols and the experimental conditions used in the present study have been previously illustrated in detail.^{20, 21} However, to facilitate drug diffusion into the wells in antagonist type experiments, the present studies were performed at 37 °C and three cycles of mixing (25 KI from each well moved up and down 3 times) were performed immediately after antagonist injection to the wells. In addition, inhibition response curve to putative antagonists were performed against the stimulatory effect of 30 nM NPS.

NPS increased the intracellular calcium concentrations in a concentration-dependent manner with pEC₅₀ and E_{max} values of 8.32 and 295% over basal, respectively (Table 1). Confirming previous findings,²⁰ [D-Val⁵]NPS did not evoke any effect per se but inhibited in a concentration dependent manner the stimulatory effect of 30 nM NPS, thus behaving as NPSR antagonist. A pK_B value of 6.54 was derived from these experiments. The replacement of the isopropyl group (as in Val) with sec-butyl group (compound **1** and **2**) produced a similar moderate reduction of potency independently from the side chain chiral center. Similar results were obtained substituting a methyl of the isopropyl group with an oxydril function (compound **3** and **4**). In this latter case, the side chain chiral center seems to exert an effect on peptide efficacy since [D-allo-Thr⁵]NPS behaves as a pure NPSR antagonist while [DThr⁵]NPS as a low efficacy partial agonist. However the difference in efficacy between **3** and **4** ([DThr⁵]NPS $\alpha = 0.08$; [D-allo-Thr⁵]NPS $\alpha = 0$) is too little to be meaningful. A linear three carbon atoms side chain (compound **5**) produced an analogue that behaved as a NPSR low efficacy partial agonist 10-fold less potent than [D-Val⁵]NPS. All together these findings indicated that the isopropyl moiety is highly important for NPSR antagonist binding and that the replacement of one of its methyl groups with ethyl or oxydril functions produced a reduction of potency. Moreover, the three carbon atoms of the D-Val side chain must have a ramified (isopropyl) rather than linear (n-propyl, as in compound **5**) shape; in fact the latter generates a clear reduction of peptide potency. The introduction in position 5 of a cyclohexyl or methyl-cyclohexyl moiety (compound **6** and **7**) generated inactive derivatives while the introduction of a phenyl ring (compound **8**) produced only a 3-fold reduction in potency compared to the isopropyl moiety of [D-Val⁵]NPS. These data suggest that the increase in the side chain size (as in compound **6** and **7**) decreases peptide potency. This is further suggested by the results obtained with compound **8**. In fact, the aromaticity of the phenyl ring of **8** reduced the side chain size and changed its shape compared with the cyclohexyl moiety of **6** and this may explain the moderate potency of [D-Phg⁵]NPS compared to the inactivity of [cyclohexyl-D-Gly⁵]NPS. Next, the effect of the insertion in the D-Val⁵ isopropyl moiety of a CH₃ (compound **9**) or SH (compound **10**) group was evaluated. In both cases the chemical change did not modify the pharmacological activity of the peptides i.e. they behaved as pure antagonists, with a 3 fold increase of potency. Finally, the insertion of a carbon atom between the tBu moiety and the peptide backbone (compound **11**) caused an important reduction of peptide potency associated with a clear increase in efficacy ([tBu-D-Gly⁵]NPS $\alpha = 0$, pK_B 7.06; [tBu-D-Ala⁵]NPS $\alpha = 0.35$, pK_B 6.32). Similar results were obtained with the isopropyl ([D-Val⁵]NPS and [D-Leu⁵]NPS²⁰). Collectively these findings indicated that a short side chain favors high potency and

pure receptor antagonism. In addition, the comparison of the effects of the side chain structures (tBu and isopropyl) clearly indicates that aliphatic branched moieties are better recognized by the NPSR receptor. The best results are obtained with the tBu moiety in which the substitution of a CH₃ with SH does not change the biological activity.

Then, [tBu-D-Gly⁵]NPS was further characterized by assessing its antagonist behavior using the classical Schild protocol and its selectivity of action over eight unrelated GPCRs. Figure 1 summarizes the data obtained by performing concentration response curves to NPS in the presence of increasing concentrations (0.1 – 10 KM) of [tBu-D-Gly⁵]NPS. This peptide produced a concentration dependent rightward shift of the concentration response curve to NPS which was however associated with a slight but significant depression of NPS maximal effects. From these experiments a pK_B values of 6.78 (CL_{95%} 6.33 – 7.23) was derived (using the equation described at pp 117 of²²). This value is close to that obtained in inhibition response curve studies (7.06, Table 1). Thus, these experiments confirmed the pure and potent antagonist properties of [tBu-D-Gly⁵]NPS. However these results are not compatible with a simple competitive interaction between [tBu-D-Gly⁵]NPS and NPS. Similar results (rightward shift associated with significant depression of agonist maximal effects) were previously reported for [D-Val⁵]NPS²⁰ which was however tested at room temperature and without the 3 cycles of mixing. Under these same experimental condition [tBu-D-Gly⁵]NPS caused a profound depression of NPS maximal effects (down to less than 50%) in a concentration dependent manner (data not shown). This depression of E_{max} was strongly reduced even if not completely eliminated performing the experiments at 37 °C and introducing the 3 cycles of mixing. Collectively these findings suggest that the depression of NPS E_{max} caused by NPSR antagonists in the calcium mobilization assay may likely derive from hemiequilibrium conditions due to lack of stirring²² rather than from a real insurmountable type of antagonism.

In order to get information about the selectivity of action of [tBu-D-Gly⁵]NPS the peptide was evaluated as agonist and antagonist in calcium mobilization experiments performed using eight unrelated human G protein coupled receptors (Table 2). These included the native PAR2 receptors expressed in A549 cells and the recombinant NK-1, B₂, and UT receptors expressed in CHO cells. Moreover, this investigation was extended to nociceptin/orphanin FQ peptide receptor (NOP) and classical opioid receptors forced to couple with the calcium pathway by the chimeric protein α_{qis} ^{23, 24}. [tBu-D-Gly⁵]NPS up to 10 KM neither stimulated calcium mobilization in these cells nor affected the stimulatory effects elicited by the reference receptor agonists (Table 2). These results suggest that [tBu-D-Gly⁵]NPS behaves as a selective NPSR antagonist. However, the panel of receptors investigated is probably too limited to draw final conclusions about the compound selectivity at this time.

Collectively the present findings demonstrated that the peptide [tBu-D-Gly⁵]NPS behaves as pure, potent (pK_B ≈ 7) and selective NPSR antagonist. This molecule should be compared to the NPSR antagonists so far available which are the moderate potency (pK_B ≈ 6.5) peptide antagonists [D-Cys(tBu)⁵]NPS²¹ and [D-Val⁵]NPS²⁰ and the highly potent (pK_B ≈ 7.5) bicyclic piperazine derivative SHA 68.²⁵ The antagonist properties of these molecules have been already confirmed in in vivo studies^{5, 20, 21, 25, 26} while the evaluation of [tBu-D-Gly⁵]NPS in vivo actions is under way in our laboratories.

In conclusion, the present study i) confirmed previous indications that the D relative configuration of amino acid residues at position 5 of NPS promotes antagonist activity, ii) indicated that the peptide antagonist potency is inversely related to the D-Xaa⁵ side chain size, and iii) demonstrated that the tBu (and its sulphhydryl derivative) directly linked to the C α carbon atom is the best chemical moiety for increasing antagonist potency. [tBu-D-Gly⁵]NPS and [D-Pen⁵]NPS identified in the context of the present study represent the most potent NPSR peptide antagonists so far identified.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by funds from the University of Ferrara (FAR grants to GC and SS), the Italian Ministry of the University (PRIN grant to GC and SS) and from the National Institute of Mental Health (grant to RKR).

References

1. Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, Brucher FA, Zeng J, Ly NK, Henriksen SJ, de Lecea L, Civelli O. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. *Neuron* 2004;43:487–497. [PubMed: 15312648]
2. Rizzi A, Vergura R, Marzola G, Ruzza C, Guerrini R, Salvadori S, Regoli D, Calo G. Neuropeptide S is a stimulatory anxiolytic agent: a behavioural study in mice. *Br J Pharmacol* 2008;154:471–479. [PubMed: 18376418]
3. Leonard SK, Dwyer JM, Sukoff Rizzo SJ, Platt B, Logue SF, Neal SJ, Malberg JE, Beyer CE, Schechter LE, Rosenzweig-Lipson S, Ring RH. Pharmacology of neuropeptide S in mice: therapeutic relevance to anxiety disorders. *Psychopharmacology (Berl)* 2008;197:601–611. [PubMed: 18311561]
4. Vitale G, Filafiero M, Ruggieri V, Pennella S, Frigeri C, Rizzi A, Guerrini R, Calo G. Anxiolytic-like effect of neuropeptide S in the rat defensive burying. *Peptides* 2008;29:2286–2291. [PubMed: 18793688]
5. Jungling K, Seidenbecher T, Sosulina L, Lesting J, Sangha S, Clark SD, Okamura N, Duangdao DM, Xu YL, Reinscheid RK, Pape HC. Neuropeptide S-mediated control of fear expression and extinction: role of intercalated GABAergic neurons in the amygdala. *Neuron* 2008;59:298–310. [PubMed: 18667157]
6. Smith KL, Patterson M, Dhillo WS, Patel SR, Semjonous NM, Gardiner JV, Ghatei MA, Bloom SR. Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. *Endocrinology* 2006;147:3510–3518. [PubMed: 16574794]
7. Beck B, Fernet B, Stricker-Krongrad A. Peptide S is a novel potent inhibitor of voluntary and fast-induced food intake in rats. *Biochem Biophys Res Commun* 2005;332:859–865. [PubMed: 15919054]
8. Cline MA, Godlove DC, Nandar W, Bowden CN, Prall BC. Anorexigenic effects of central neuropeptide S involve the hypothalamus in chicks (*Gallus gallus*). *Comp Biochem Physiol A Mol Integr Physiol* 2007;148:657–663. [PubMed: 17884648]
9. Meis S, Bergado-Acosta JR, Yanagawa Y, Obata K, Stork O, Munsch T. Identification of a neuropeptide S responsive circuitry shaping amygdala activity via the endopiriform nucleus. *PLoS ONE* 2008;3:e2695. [PubMed: 18628994]
10. Badia-Elder NE, Henderson AN, Bertholomey ML, Dodge NC, Stewart RB. The Effects of Neuropeptide S on Ethanol Drinking and Other Related Behaviors in Alcohol-Preferring and -Nonpreferring Rats. *Alcohol Clin Exp Res* 2008;32:1380–1387. [PubMed: 18564106]
11. Li W, Gao YH, Chang M, Peng YL, Yao J, Han RW, Wang R. Neuropeptide S inhibits the acquisition and the expression of conditioned place preference to morphine in mice. *Peptides* 2009;30:234–240. [PubMed: 18992779]
12. Paneda C, Huitron-Resendiz S, Frago LM, Chowen JA, Picetti R, de Lecea L, Roberts AJ. Neuropeptide S reinstates cocaine-seeking behavior and increases locomotor activity through corticotropin-releasing factor receptor 1 in mice. *J Neurosci* 2009;29:4155–4161. [PubMed: 19339610]
13. Cannella N, Economidou D, Kallupi M, Stopponi S, Heilig M, Massi M, Ciccocioppo R. Persistent Increase of Alcohol-Seeking Evoked by Neuropeptide S: an Effect Mediated by the Hypothalamic Hypocretin System. *Neuropsychopharmacology*. 2009in press
14. Bernier V, Stocco R, Bogusky MJ, Joyce JG, Parachoniak C, Grenier K, Arget M, Mathieu MC, O'Neill GP, Slipetz D, Crackower MA, Tan CM, Therien AG. Structure-function relationships in the neuropeptide S receptor: molecular consequences of the asthma-associated mutation N107I. *J Biol Chem* 2006;281:24704–24712. [PubMed: 16790440]

15. Reinscheid RK, Xu YL, Okamura N, Zeng J, Chung S, Pai R, Wang Z, Civelli O. Pharmacological Characterization of Human and Murine Neuropeptide S Receptor Variants. *J Pharmacol Exp Ther* 2005;315:1338–1345. [PubMed: 16144971]
16. Roth AL, Marzola E, Rizzi A, Arduin M, Trapella C, Corti C, Vergura R, Martinelli P, Salvadori S, Regoli D, Corsi M, Cavanni P, Calo G, Guerrini R. Structure-activity studies on neuropeptide S: identification of the amino acid residues crucial for receptor activation. *J Biol Chem* 2006;281:20809–20816. [PubMed: 16720571]
17. Tancredi T, Guerrini R, Marzola E, Trapella C, Calo G, Regoli D, Reinscheid RK, Camarda V, Salvadori S, Temussi PA. Conformation-Activity Relationship of Neuropeptide S and Some Structural Mutants: Helicity Affects Their Interaction with the Receptor. *J Med Chem* 2007;50:4501–4508. [PubMed: 17696420]
18. Camarda V, Trapella C, Calo G, Guerrini R, Rizzi A, Ruzza C, Fiorini S, Marzola E, Reinscheid RK, Regoli D, Salvadori S. Synthesis and biological activity of human neuropeptide S analogues modified in position 2. *J Med Chem* 2008;51:655–658. [PubMed: 18181564]
19. Camarda V, Trapella C, Calo G, Guerrini R, Rizzi A, Ruzza C, Fiorini S, Marzola E, Reinscheid RK, Regoli D, Salvadori S. Structure activity study at position 3 and 4 of human neuropeptide S. *Bioorg Med Chem* 2008;16:8841–8845. [PubMed: 18793857]
20. Guerrini R, Camarda V, Trapella C, Calo G, Rizzi A, Ruzza C, Fiorini S, Marzola E, Reinscheid R, Regoli D, Salvadori S. Synthesis and biological activity of human neuropeptide S analogues modified in position 5: identification of potent and pure NPS receptor antagonists. *J Med Chem* 2009;52:524–529. [PubMed: 19113861]
21. Camarda V, Rizzi A, Ruzza C, Zucchini S, Marzola G, Marzola E, Guerrini R, Salvadori S, Reinscheid RK, Regoli D, Calo G. In vitro and in vivo pharmacological characterization of the neuropeptides S receptor antagonist [D-Cys(tBu)5]NPS. *J Pharmacol Exp Ther* 2009;328:549–555. [PubMed: 18971372]
22. Kenakin, T. *A Pharmacology Primer*. Vol. 2. Elsevier Academic Press; San Diego: 2006.
23. Coward P, Chan SD, Wada HG, Humphries GM, Conklin BR. Chimeric G proteins allow a high-throughput signaling assay of Gi-coupled receptors. *Anal Biochem* 1999;270:242–248. [PubMed: 10334841]
24. Camarda V, Fischetti C, Anzellotti N, Molinari P, Ambrosio C, Kostenis E, Regoli D, Trapella C, Guerrini R, Salvadori S, Calo G. Pharmacological profile of NOP receptors coupled with calcium signaling via the chimeric protein Ga_{q15}. *Naunyn Schmiedeberg Arch Pharmacol* 2009;379:599–607. [PubMed: 19183962]
25. Okamura N, Habay SA, Zeng J, Chamberlin AR, Reinscheid RK. Synthesis and pharmacological in vitro and in vivo profile of 3-oxo-1,1-diphenyl-tetrahydro-oxazolo[3,4-a]pyrazine-7-carboxylic acid 4-fluoro-benzylamide (SHA 68), a selective antagonist of the neuropeptide S receptor. *J Pharmacol Exp Ther* 2008;325:893–901. [PubMed: 18337476]
26. Li W, Chang M, Peng YL, Gao YH, Zhang JN, Han RW, Wang R. Neuropeptide S produces antinociceptive effect at the supraspinal level in mice. *Regul Pept*. 2009;in press

Figure 1.

Concentration response curve to NPS obtained in the absence (control) and in presence of increasing concentrations of [tBu-D-Gly⁵]NPS in calcium mobilization experiments performed in HEK293_{mNPSR} cells. Data are mean $\pm$ sem of 4 separate experiments made in duplicate.

Effects of NPS and [D-Xaa⁵]NPS analogues in HEK293 cells expressing the mouse NPSR.

Table 1

Xaa ⁵	Chemical formula	Name	Agonist		Antagonist	
			pEC ₅₀ (CI _{95%})	E _{max} ± sem	pK _B (CI _{95%})
	Gly	8.32 (8.00 - 8.64)	295 ± 22%	-
	D-Val	inactive		6.54 (5.99 - 6.99)	

Name	Agonist pEC ₅₀ (CL _{95%})	E _{max} ± sem	Antagonist pK _B (CL _{95%})
D-Ile		inactive	5.72 (5.10 – 6.34)

Xaa⁵

Chemical formula

Xaa ⁵	Chemical formula	Name	Agonist		Antagonist	
			pEC ₅₀ (CL _{95%})	E _{max} ± sem	pK _B (CL _{95%})
	D-allo-Ile		inactive	6.04 (5.52 – 6.56)	

Xaa⁵

Chemical formula

Name	Agonist		Antagonist	
	pEC ₅₀ (CL _{95%})	E _{max} ± sem	pK _B (CL _{95%})	
D-Thr	6.10 (5.79 – 6.41)	25 ± 5% *	6.09 (5.79 – 6.39)	

Chemical formula	Name	Agonist		Antagonist	
		pEC ₅₀ (CL _{95%})	E _{max} ± sem	pK _B (CL _{95%})	
	D-allo-Thr		inactive	6.04 (5.51 – 6.57)	

Xaa⁵

Xaa⁵

Chemical formula

Name	Agonist		Antagonist	
	pEC ₅₀ (CI _{95%})	E _{max} ± sem	pK _B (CI _{95%})	
D-Nva	5.57 (5.02 – 6.12)	24 ± 7% *	5.68 (5.38 – 5.98)	
cyclohexyl-D-Gly	inactive		< 5	
D-Cha	inactive		< 5	
D-Phg	6.18 (5.81 – 6.55)	15 ± 2% *	6.12 (5.81 – 6.43)	
tBu-D-Gly	inactive		7.06 (6.48 – 7.64)	

Xaa ⁵	Chemical formula	Name	Agonist		Antagonist	
			pEC ₅₀ (CL _{95%})	E _{max} ± sem	pK _B (CL _{95%})
	D-Pen		inactive	7.08 (6.46 – 7.70)	

Xaa ⁵	Chemical formula	Agonist		Antagonist	
		pEC ₅₀ (CI _{95%})	E _{max} ± sem	pK _B (CI _{95%})	
		5.78 (5.06 – 6.50)	104 ± 2% *	6.32 (5.63 – 7.01)

J Med Chem. Author manuscript; available in PMC 2010 July 9

e logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect.

CL-95% : 95% confidence limits.

E_{max} : the maximal effect elicited by the agonist expressed as % over the baseline.

sem: standard error of the mean.

inactive: inactive up to 10 KM.

The antagonist potencies (pK_B) of the peptides were assessed in inhibition response curves against the stimulatory effect elicited by 30 nM NPS.

* p < 0.05 vs NPS, according to one-way ANOVA followed by the Dunnett test. Data are means of at least 4 separate experiments made in duplicate.

Table 2
Selectivity profile of [tBu-D-Gly⁵]NPS at eight different human G-protein coupled receptors.

Cell line	Receptor	Agonist	control			[tBu-D-Gly ⁵]NPS 10 KM	
			pEC ₅₀	E _{max} ± sem	pEC ₅₀	E _{max} ± sem	E _{max} ± sem
CHO	recombinant NK-1	substance P	10.24 (9.86–10.62)	122 ± 15%	10.31 (9.94–10.68)	113 ± 11%	
CHO	recombinant B ₂	bradykinin	10.31 (10.25–10.37)	125 ± 2%	10.47 (9.90–11.04)	115 ± 11%	
CHO	recombinant UT	urotensin-II	8.39 (7.50–8.88)	224 ± 21%	8.46 (8.19–8.73)	234 ± 19%	
CHO-α _{q15}	recombinant MOP	dermorphin	7.98 (7.85–8.11)	186 ± 11%	7.96 (7.71–8.21)	186 ± 15%	
CHO-α _{q15}	recombinant DOP	DPDPE	8.53 (8.23–8.73)	169 ± 26%	8.71 (8.65–8.77)	177 ± 11%	
CHO-α _{q15}	recombinant KOP	dynorphin A	8.20 (7.81–8.59)	143 ± 12%	8.63 (8.33–8.99)	139 ± 13%	
CHO-α _{q15}	recombinant NOP	N/OFQ	9.41 (9.26–9.56)	231 ± 12%	9.55 (9.11–9.99)	207 ± 30%	
A549	native PAR2	SLIGKV-NH ₂	4.55 (4.06–5.04)	449 ± 25%	4.50 (3.96–5.04)	445 ± 30%	

Data are mean ± sem of three separate experiments performed in duplicate. The chimeric protein α_{q15} was used to force NOP and classical opioid receptors to couple with the calcium pathway. N/OFQ: nociceptin/orphanin FQ.