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Comparison of the effects of [Phe$^1\Psi$(CH$_2$-NH)Gly^2]Nociceptin (1-13)NH$_2$ in rat brain, rat vas deferens and CHO cells expressing recombinant human nociceptin receptors

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1 Nociceptin(NC) is the endogenous ligand for the opioid receptor like-1 receptor (NC-receptor). [Phe$^1\Psi$(CH$_2$-NH)Gly^2]Nociceptin(1-13)NH$_2$ ([F/G]NC(1-13)NH$_2$) has been reported to antagonize NC actions in peripheral guinea-pig and mouse tissues. In this study, we investigated the effects of a range of NC C-terminal truncated fragments and [F/G]NC(1-13)NH$_2$ on NC receptor binding, glutamate release from rat cerebrocortical slices (rCX), inhibition of cyclic AMP accumulation in CHO cells expressing the NC receptor (CHO$_{NCR}$) and electrically evoked contractions of the rat vas deferens (rVD).

2 In radioligand binding assays, a range of ligands inhibited [^{125}I]-Tyr^{14}-NC binding in membranes from rCX and CHO$_{NCR}$ cells. As the peptide was truncated there was a general decline in pK$_i$. [F/G]NC(1-13)NH$_2$ was as potent as NC(1-13)NH$_2$.

3 The order of potency for NC fragments to inhibit cyclic AMP accumulation in whole CHO$_{NCR}$ cells was NCNH$_2$ $\geqslant$ NC = NC(1-13)NH$_2$ > NC(1-12)NH$_2$ > > NC(1-11)NH$_2$. [F/G]NC(1-13)NH$_2$ was a full agonist with a pEC$_{50}$ value of 8.65.

4 NCNH$_2$ and [F/G]NC(1-13)NH$_2$ both inhibited K$^+$ evoked glutamate release from rCX with pEC$_{50}$ and maximum inhibition of 8.16, 48.5 $\pm$ 4.9% and 7.39, 58.9 $\pm$ 6.8% respectively.

5 In rVD NC inhibited electrically evoked contractions with a pEC$_{50}$ of 6.63. Although [F/G]NC(1-13)NH$_2$, displayed a small (instrinsic activity $\alpha = 0.19$) but consistent residual agonist activity, it acted as a competitive antagonist (pA$_2$ 6.76) in the rVD.

6 The differences between [F/G]NC(1-13)NH$_2$ action on central and peripheral NC signalling could be explained if [F/G]NC(1-13)NH$_2$ was a partial agonist with high strength of coupling in the CNS and low in the periphery. An alternative explanation could be the existence of central and peripheral receptor isoforms.

Keywords: Nociceptin/orphanin FQ; nociceptin receptor; binding; cyclic AMP; glutamate release; vas deferens; rat

Abbreviations: CHO, Chinese hamster ovary; NC, Nociceptin/orphanin FQ; PTX, pertussis toxin

Introduction

Nociceptin/orphanin FQ (NC) is the endogenous ligand for the guanine nucleotide-binding regulatory protein (G-protein) coupled opioid receptor like-1 (ORL-1) receptor (Meunier *et al*., 1995; Reinscheid *et al*., 1995). In the remainder of this manuscript we will use the terminology NC-receptor. Although this receptor has significant (63–65%) sequence homology (see Meunier, 1997 for review) to OP$_1$, OP$_2$ and OP$_3$ opioid receptors it does not bind traditional opioid ligands (see Meunier, 1997 for review). Moreover, NC does not bind to the classical opioid receptors with high affinity (Meunier *et al*., 1995; Reinscheid *et al*., 1995), suggesting a distinct divergence between the NC/NC-receptor system and the opioid systems. NC produced hyperalgesia and/or antiopioid actions when injected intracerebroventricularly whilst it exhibits antinociceptive effects when administered intrathecally (see Meunier, 1997 for review). Despite these observations, the cellular actions of NC are similar to those of opioids. NC activates the NC-receptor to either inhibit adenylyl cyclase (Meunier *et al*., 1995; Reinscheid *et al*., 1995) and/or voltage-gated calcium channels (Connor *et al*., 1996b; Abdulla & Smith, 1997) or to stimulate an inwardly rectifying potassium conductance (Matthes *et al*., 1996; Connor *et al*., 1996a; Vaughan & Christie, 1996). These actions are expected to reduce neuronal excitability and neurotransmitter release. Indeed, we have recently reported NC induced inhibition of K$^+$ evoked glutamate release from rat cerebrocortical slices (Nicol *et al*., 1996) and others also reported inhibition of glutamatergic transmission (Faber *et al*., 1996; Vaughan *et al*., 1997), GABA-ergic transmission (Vaughan *et al*., 1997), dopamine (Murphy *et al*., 1996), noradrenaline (Schlicker *et al*., 1998), tachykinin (Giuliani & Maggi, 1996; Helyes *et al*., 1997) and acetylcholine (Patel *et al*., 1997; Neal *et al*., 1997) release.

The transcript encoding the NC-receptor is found not only in the central nervous system (Darland & Grandy, 1998) but also in peripheral tissues such as rat intestine, skeletal muscle, vas deferens, spleen and lymphocytes (Wang *et al*., 1994; Peluso *et al*., 1998; Wick *et al*., 1995). NC is reported to inhibit electrically induced contractions in several peripheral isolated preparations (see Meunier, 1997 for review).

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In most studies, naloxone did not antagonize the effects of NC excluding an activation of OP_1, OP_2 and OP_3 opioid receptors. However, it had been difficult to demonstrate the direct activation of the NC-receptor by NC due to the absence of a selective antagonist for this receptor. Recently, a selective competitive antagonist for the NC-receptor, [Phe$^1\Psi$(CH$_2$-NH)Gly^2]nociceptin(1-13)NH$_2$ ([F/G]NC(1-13)NH$_2$) has been reported by three of us (GC, RG, RB, Guerrini *et al*., 1998; Calo' *et al*., 1998a).

The objective of this study was to investigate the pharmacological properties of a range of NC C-terminal truncated fragments that have already been studied in different models (Reinscheid *et al*., 1996; Dooley & Houghten, 1996; Guerrini *et al*., 1997; Calo' *et al*., 1997; Rossi *et al*., 1997; Butour *et al*., 1997) and [F/G]NC(1-13)NH$_2$ using preparations expressing NC-receptors from the central nervous system and periphery. We describe binding characteristics of these agents in membranes from rat cerebrocortex and CHO cells expressing the recombinant human NC-receptor (CHO_{NCR}). In functional studies, effects on cyclic AMP accumulation and K^+ evoked glutamate release (Nicol *et al*., 1996) were examined in whole CHO_{NCR} cells and rat cerebrocortical slices, respectively. The effects of nociceptin and [F/G]NC(1-13)NH$_2$ on electrically stimulated rat vas deferens were also tested to enable a direct comparison to be made.

Methods

Sources of chemicals, reagents and equipment

The chemicals and their sources were as follows; NC, its amidated fragments and [F/G]NC(1-13)NH$_2$ were prepared at one of our institutes as previously described (Guerrini *et al*., 1997, Calo' *et al*., 1998a). Cell culture medium (DMEM, F12), foetal calf serum, glutamine, hygromycin B and G418 were from Gibco (Paisley, U.K.). [^{125}I]-Tyr^{14}-nociceptin (2000 Ci mmol^{-1}) and [2,8-^{3}H]-cAMP (28.4 Ci mmol^{-1}) were from Amersham (Little Chalfont, U.K.) and NEN DuPont (Boston, MA, U.S.A.), respectively. HEPES was from USB (Cleveland, OH, U.S.A.) and $MgSO_4$ was from Fisons Scientific (Loughborough, U.K.). Phosphoramidon was from peptide institute (Osaka, Japan). All other reagents were from Sigma Chemical Co. (Poole, U.K.). CHO cells stably expressing the NCR receptor, CHO_{NCR} (transfected using pCIN5) were obtained from Dr F Marshall and Mrs N Bevan of Glaxo-Wellcome, Stevenage, Herts, U.K.

[^{125}I]-Tyr^{14}-NC binding

Female Wistar rats (250–300 g) were decapitated following cervical dislocation. The brain was removed and rapidly transferred to ice-cold buffer (Tris-HCl 50 mM, pH 7.4) and the cerebrocortex dissected. CHO_{NCR} cells were maintained in DMEM:F12 (50 : 50) containing 5% foetal calf serum, 2 mM glutamine, 200 μg ml^{-1} hygromycin B and 200 μg ml^{-1} G418. Cultures were maintained at 37°C in 5% CO_2/humidified air. When confluency was reached (3–4 days), cells were harvested for use by the addition of 0.9% saline containing HEPES (10 mM) and EDTA (0.02%). These tissues were homogenized on ice and the homogenate was centrifuged at 13,500 r.p.m for 10 min at 4°C and the pellet resuspended in Tris-HCl buffer. This procedure was repeated twice more. Membranes were prepared fresh each day. All binding assays were performed in 1 ml of buffer (Tris-HCl 50 mM, $MgSO_4$ 5 mM containing 30 μM of peptidase inhibitors; captopril, amastatin, bestatin and phosphoramidon and Bovine serum albumin (BSA) 0.5%, pH 7.4) for 30 min at room temperature with 1–2 pM [^{125}I]-Tyr^{14}-NC and 50 μg of membrane protein from rat cerebrocortex and 3–4 pM [^{125}I]-Tyr^{14}-NC and $\sim$2 μg membrane protein from CHO_{NCR} cells. Non-specific binding (NSB) was defined in the presence of 10^{-6}M nociceptin. These optimal incubation conditions were determined empirically. Following incubation, bound and free radioactivities were separated by vacuum filtration using a Brandel cell harvester. Harvester papers (Whatman GF/B) were soaked in polyethylenimine (0.5%) to reduce NSB, and loaded onto the harvester wet. For determination of the maximal binding capacity (B_{max}) and the equilibrium dissociation constant (K_D), a pseudo-isotope dilution study was performed (i.e., competition between NC and NC with ^{125}I-Tyr^{14} substitution as described by Ardati *et al*., 1997). In both pseudo-isotope dilution and displacement studies, approximately 1–4 pM of [^{125}I]-Tyr^{14}-NC with increasing concentrations of unlabelled displacers were used.

Inhibition of cyclic AMP accumulation

For the measurement of cyclic AMP, whole CHO_{NCR} cells were incubated in 0.3 ml Krebs-HEPES buffer containing BSA (0.5%), peptidase inhibitors (30 μM; captopril, amastatin, bestatin, phosphoramidon) 1-isobutyl-4-methylxanthine (IBMX; 1 mM) and forskolin (1 μM). After 15 min at 37°C, the reaction was terminated by the addition of 20 μl HCl (10 M). Following neutralization by the addition of 20 μl NaOH (10 M) and 180 μl of Tris-HCl (1 M, pH 7.4) and centrifugation (13,000 $\times$ *g* for 2 min), cyclic AMP was measured in the supernatant by a protein-binding assay using a binding protein from bovine adrenal cortex (Okawa *et al*., 1998). In some experiments, untransfected CHO cells (CHOwt) and CHO_{NCR} cells preincubated with 100 ng ml^{-1} pertussis toxin (PTX) in culture medium for 24 h were used to examine the involvement of the NC-receptor and pertussis toxin sensitive G proteins, respectively.

Effects on glutamate release from rat cerebrocortical slices

This was performed as described previously (Nicol *et al*., 1996). Briefly, female Wistar rats (250–300 g) were decapitated following cervical dislocation. The brain was removed and rapidly transferred to ice-cold oxygenated (95% O_2 and 5% CO_2) Krebs buffer, pH 7.4. Slices were cut and washed three times in fresh Krebs buffer prior to agitation in a shaking water bath at 37°C for 40 min; 1 ml of gravity-packed slices were pipetted into a perfusion chamber. Slices were perfused at 37°C for 60 min at 1 ml min^{-1} prior to collection of 2 min fractions. A 2 min pulse of 46 mM K^+ (Na^+ adjusted) was applied (S_1) following 6 min of perfusion. Slices were perfused for a further 30 min, prior to the second application of a 2 min pulse of 46 mM K^+ (S_2). Perfusate glutamate concentrations were measured fluorimetrically, expressed relative to the mean of the first three basal samples and S_2/S_1 ratios were calculated. To examine the effects of NC-receptor ligands on glutamate release they were applied immediately after S_1 until the end of the experiment (also present during S_2) in various combinations.

Effects on electrically evoked contractions of the rat vas deferens (rVD)

Male Sprague Dawley rats (350–400 g) were decapitated, the vas deferens isolated and suspended in 10 ml organ baths

containing Krebs solution ((in mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 1.8, glucose 10) oxygenated with 95% O_2 and 5% CO_2 at 37°C. In some experiments the peptidase inhibitor cocktail (30 μM; captopril, amastatin, bestatin, phosphoramidon) was added to the incubation buffer 30 min before peptide application. A resting tension of 1 g was applied to the tissue. The vas deferens were continuously stimulated through two platinum ring electrodes with supramaximal voltage rectangular pulses of 1 msec duration and 0.1 Hz frequency. The electrically evoked contractions were measured isotonically with a strain gauge transducer (Basile 7006) and recorded on a Linseis multichannel chart recorder (model 2005). After an equilibration period of about 60 min the contractions induced by electrical field stimulation (EFS) were stable; at this time, cumulative concentration-response curves (crc) to NC were performed (0.5 log unit steps). When required, the NC receptor antagonist, [F/G]NC(1-13)NH_2, or the non selective opioid receptor antagonist, naloxone, were added to the medium 15 min before performing crc to NC.

Data analysis

Data are presented as the mean ± s.e.mean (n, independent experiments). In binding studies, B_{max} and pK_D were obtained from pseudo-isotope dilution studies. Concentration of displacers producing 50% displacement of specific binding (pIC_{50}) was corrected for the competing mass of [^{125}I]-Tyr^{14}-NC to yield pK_i. All curve fitting was performed using PRISM-V2.0 (GraphPAD software, San Diego, CA, U.S.A.). Statistical comparisons of paired samples were made using Freidmans analysis of variance and Wilcoxon Rank sum test as appropriate and considered significant when $P<0.05$.

Results

[^{125}I]-Tyr^{14}-NC binding

The binding of NC assessed using [^{125}I]-Tyr^{14}-NC (pseudo-isotope dilution) was concentration-dependent and saturable. B_{max} (fmol mg protein^{-1}) and pK_D values in rat cerebrocortex (from Okawa *et al*., 1998) and CHO_{NCR} cells were 179.7 ± 15.3, 10.26 ± 0.09 ($n=5$) and 1716 ± 363, 9.70 ± 0.23 respectively ($n=3$). Binding slope factors in both membrane preparations were close to unity (1.04 ± 0.05 and 0.93 ± 0.07 in rat cerebrocortical and CHO_{NCR} membranes, respectively). NC and a range of NC fragments/analogues examined all produced concentration-dependent inhibition of [^{125}I]-Tyr^{14}-NC binding in both membrane preparations, with pK_i values shown in Table 1. There were no differences between NC and $NCNH_2$ in either preparation. In both preparations as the peptide was truncated from 13 to 9 amino acids there was a general decline in pK_i. There were no significant differences between [F/G]NC(1-13)NH_2 and NC(1-13)NH_2 in either preparation ($P<0.05$) (Figure 1). In both preparations naloxone <10 μM did not inhibit [^{125}I]-Tyr^{14}-NC binding (data not shown).

Table 1 Inhibition of [^{125}I]-Tyr^{14}-nociceptin (for graphical representation see Figure 1)

	Binding pK_i	
	Cerebrocortex	*CHO_{NCR} cells*
NC	10.26 ± 0.09	9.70 ± 0.23
$NCNH_2$	9.60 ± 0.02	9.65 ± 0.07
NC(1–13)NH_2	9.69 ± 0.06	10.36 ± 0.14
NC(1–12)NH_2	9.40 ± 0.06	8.75 ± 0.11
NC(1–11)NH_2	8.49 ± 0.02	8.24 ± 0.08
NC(1–9)NH_2	7.80 ± 0.08	8.13 ± 0.08
[F/G]NC(1–13)NH_2	9.35 ± 0.01	10.20 ± 0.09

Data are mean ± s.e.mean ($n=4–5$).

Inhibition of cyclic AMP accumulation

All agents examined inhibited forskolin-stimulated cyclic AMP accumulation in CHO_{NCR} cells in a concentration-dependent manner (Figure 2) with pEC_{50} and maximal inhibition values shown in Table 2. In experiments using CHOwt cells and CHO_{NCR} cells preincubated with PTX, neither NC nor [F/G]NC(1-13)NH_2 inhibited forskolin stimulated cyclic AMP accumulation (Figure 3). These results demonstrate the

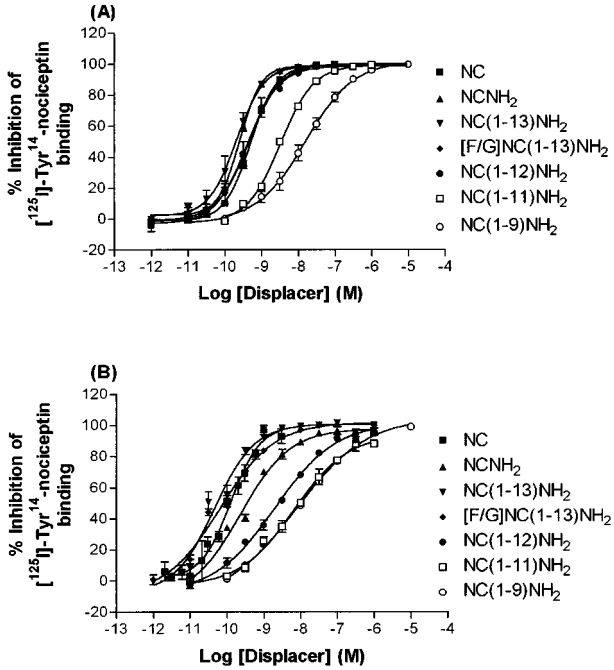

Figure 1 Inhibition of [^{125}I]-Tyr^{14}-nociceptin binding by nociceptin fragments and [F/G]NC(1-13)NH_2 in membranes from rat cerebrocortex (A) and from CHO cells expressing the human nociceptin receptor (B). All agents produced dose-dependent inhibition of [^{125}I]-Tyr^{14}-nociceptin binding. For pK_i values, see Table 1, ($n=4–5$).

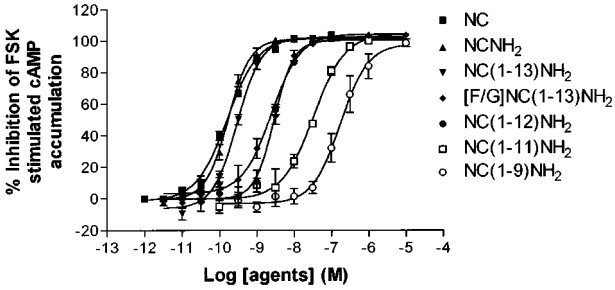

Figure 2 Effects of nociceptin fragments and [F/G]NC(1-13)NH_2 on forskolin stimulated cyclic AMP accumulation in whole CHO cells expressing the human nociceptin receptor. All agents produced dose-dependent inhibition of cyclic AMP acculumation. For pIC_{50} and I_{max} values, see Table 2, ($n=5–7$).

Table 2 Inhibition of cyclic AMP formation in CHO_{NCR} cells (for graphical representation see Figure 2)

	cyclic AMP inhibition	
	pIC_{50}	I_{max}
NC	9.78±0.03	103.4±2.6
$NCNH_2$	9.77±0.03	103.2±1.3
NC(1–13)NH_2	9.49±0.03	103.9±1.5
NC(1–12)NH_2	8.54±0.04	100.3±2.6
NC(1–11)NH_2	7.48±0.07	104.5±4.8
NC(1–9)NH_2	6.74±0.09	96.6±6.2
[F/G]NC(1–13)NH_2	8.65±0.05	104.1±2.5

Data are mean±s.e.mean (n=5–7).

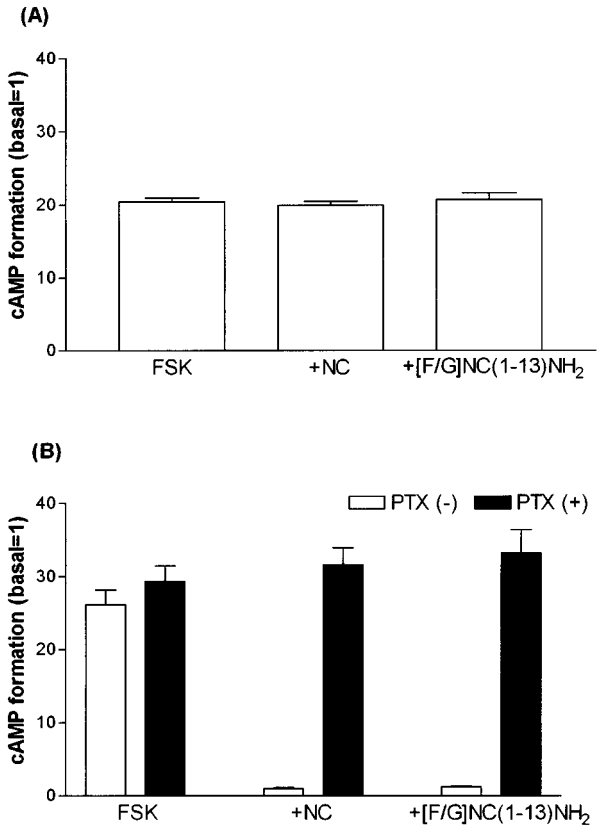

Figure 3 cyclic AMP assay carried out in untransfected CHO cells (A) and in CHO cells expressing the human nociceptin receptor preincubated with 100 ng ml^{-1} of pertussis toxin (B). Neither NC nor [F/G]NC(1-13)NH_2 inhibited forskolin stimulated cyclic AMP accumulation (n=5).

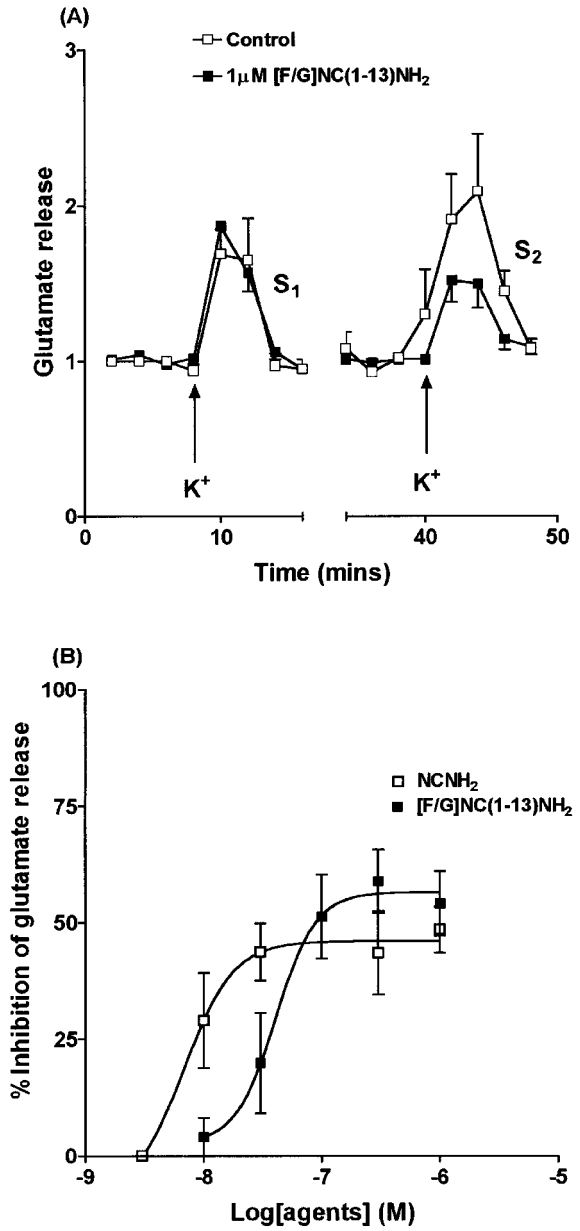

Figure 4 Effects of $NCNH_2$ and [F/G]NC(1-13)NH_2 on K^+ evoked glutamate release from rat cerebrocortical slices. Time course for effects of [F/G]NC(1-13)NH_2 is shown in (A). In (B) both agents produced dose-dependent inhibition of K^+ evoked glutamate release. (n=5 except 3 nM $NCNH_2$ where no inhibition was observed in two experiments). In (A) data are expressed relative to the mean of the first three fractions collected during S_1 or S_2.

involvement of the NC-receptor and a pertussis toxin sensitive G protein(s) in the inhibition of cyclic AMP accumulation. To examine further a possible antagonism of NC induced inhibition of cyclic AMP formation by [F/G]NC(1-13)NH_2, cells were incubated in the presence or absence of 3×10^{-9} M [F/G]NC(1-13)NH_2. This treatment produced no appreciable shift in the concentration response curve compared to control (pEC_{50}: control=9.82±0.04; +[F/G]NC(1-13)NH_2=9.67±0.06). In two additional experiments using 10^{-7} and 10^{-6} M [F/G]NC(1-13)NH_2 (and despite marked agonist activity) we were also unable to demonstrate any antagonist activity (data not shown).

Effects on glutamate release from rat cerebrocortical slices

$NCNH_2$ produced a concentration-dependent inhibition of K^+ evoked glutamate release (Figure 4) from rat cerebrocortical slices with maximal inhibition of 48.5±4.9% and a pEC_{50} of 8.16. These values were not markedly different from our previous report using NC (Nicol *et al.*, 1996). [F/G]NC(1-13)NH_2 produced a concentration-dependent inhibition of K^+ evoked glutamate release with maximal inhibition of 58.9±6.8 and a pEC_{50} of 7.39. The inhibition produced by [F/G]NC(1-13)NH_2 was naloxone insensitive (Control S_2/S_1 ratio (n=5) 0.82±0.17, +100 nM [F/G]NC(1-13)NH_2 0.48±0.07, +[F/G]NC(1-13)NH_2 and 10 μM Naloxone 0.36±0.07). In

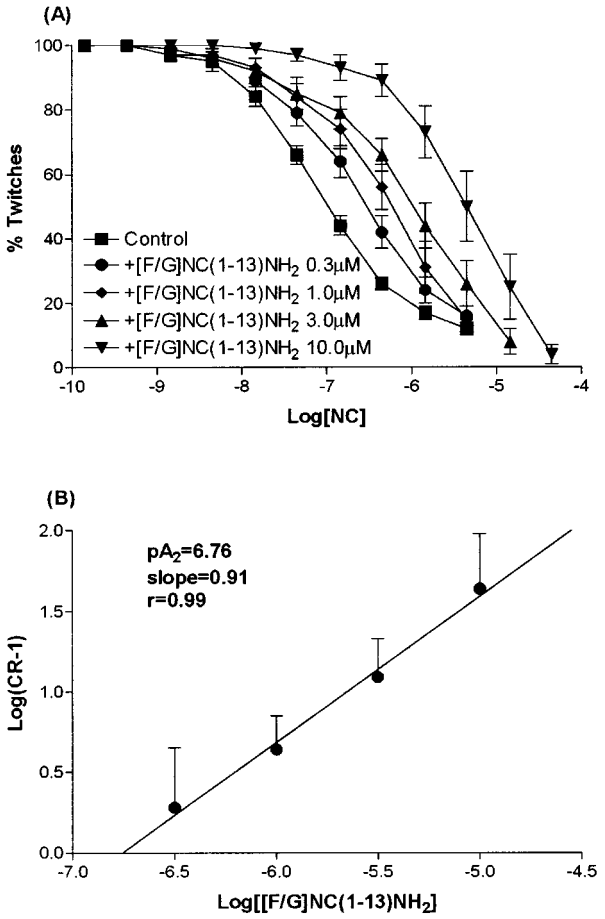

Figure 5 Effects of [F/G]NC(1-13)NH$_2$ on NC induced inhibition of contractile response in rVD. [F/G]NC(1-13)NH$_2$ produced a parallel rightward shift in the concentration response curve (A) which is depicted as a Schild plot (B). Data are mean ± s.e.mean ($n=5$). In (B) the estimated pA$_2$ for [F/G]NC(1-13)NH$_2$ was 6.76.

addition NC(1-13)NH$_2$ (the template for [F/G]NC(1-13)NH$_2$) at 300 nM inhibited release by 43.1 ± 9.9% which was similar to that of NCNH$_2$ and NC (Nicol *et al*., 1996).

Electrically stimulated rat vas deferens

NC inhibits the electrically induced contractions of the rVD with a pEC$_{50}$ value of 6.63 ± 0.2 and a maximal effect of about −80% of control values. Application of 1 μM naloxone did not modify the concentration response curve (crc) to the peptide (data not shown). Application of 1 μM [F/G]NC(1-13)NH$_2$ was followed by an inhibition of the response of the rVD which reached about −15% of control values. In the presence of the pseudopeptide the crc to NC was shifted to the right by approximately one log unit, without any significant modification of the maximal effect induced by NC. A pA$_2$ value of 6.85 was estimated for [F/G]NC(1-13)NH$_2$ by means of the Gaddum Schild equation from this series of data. These experiments were repeated in the presence of the cocktail of peptidase inhibitors (30 μM amastatin, captopril, phosphoramidon and bestatin). The inhibitors caused *per se* a slight (about −10% of control values) but consistent inhibition of the electrically induced twitches; in addition in their presence the potency of NC was significantly enhanced (pEC$_{50}$ 7.37) while that of [F/G]NC(1-13)NH$_2$ was unchanged (pA$_2$ 6.90).

We have also performed cumulative concentration response curves to [F/G]NC(1-13)NH$_2$ (1 nM – 10 μM). [F/G]NC(1-13)NH$_2$ produced a concentration dependent inhibition of electrically induced twitches with a pEC$_{50}$ of 6.84 and a maximal effect of −13 ± 5% (corresponding to an intrinsic activity α of 0.19). The antagonistic properties of [F/G]NC(1-13)NH$_2$ have also been evaluated over an extended range of concentrations (0.3 – 10 μM) to obtain data for a Schild analysis. As shown in Figure 5A, [F/G]NC(1-13)NH$_2$ displaced to the right the crc to NC in a concentration dependent manner, the curves being parallel to the control, and reaching the same maximal effects. Figure 5B shows the corresponding Schild plot which was linear ($r=0.99$) with a slope of 0.91 which was not significantly different from unity. The extrapolated pA$_2$ value was 6.76.

Discussion

Binding experiments were performed in membranes from rat cerebrocortex with the native rat brain NC-receptor and compared with those from CHO cells expressing the recombinant human NC-receptor. B_{max} and K_D values in rat cerebrocortical membranes (from our previous report, Okawa *et al*., 1998) were consistent with others using the same radioligand (Ardati *et al*., 1997). Slope factors were close to unity in both reports and suggest that [^{125}I]-Tyr^{14}-NC is binding to a single class of receptors although Mathis *et al*. (1997) reported both high and low affinity binding sites in mouse brain membranes. B_{max} in CHO$_{NCR}$ cell membranes was consistent with others (Fawzi *et al*., 1997) while it was slightly higher than those of Reinscheid *et al*. (1996) and Ardati *et al*. (1997) using the same radioligand. These differences are merely due to transfection but it is important to compare our data with those using similar expression levels. Butour *et al*. (1997) and Adapa & Toll (1997) reported values similar to ours using [^{3}H]-NC and [^{3}H]-Tyr^{14}-NC, respectively. In this study we observed binding slope factors close to unity in CHO$_{NCR}$ cell membranes, again suggesting a single binding site in accordance with others (Reinscheid *et al*., 1996; Ardati *et al*.,1997, Adapa & Toll, 1997; Mathis *et al*., 1997). Adapa & Toll (1997) conducted binding experiments using intact CHO$_{NCR}$ cells in physiological buffer similar to that used for our cyclic AMP assay. They observed high and low affinity binding sites. The latter sites were approximately two orders of magnitude weaker than that of CHO$_{NCR}$ cell membranes in Tris buffer, making up 95% of the binding. This sensitivity of NC binding to Na^+, in the same manner as opioid binding to the μ-opioid receptor (Yabaluri & Medzihradsky, 1997), must be borne in mind when comparing binding characteristics in Na^+ free buffer with functional activities in physiological media. However, comparative studies using one system may yield potency information for a range of compounds. The ability to inhibit [^{125}I]-Tyr^{14}-NC binding was similar for NC, NCNH$_2$, [F/G]NC(1-13)NH$_2$, NC(1-13)NH$_2$ and NC(1-12)NH$_2$ in membranes from both rat cerebrocortex and CHO$_{NCR}$ cells. Among NC fragments tested, NC(1-11)NH$_2$ and NC(1-9)NH$_2$ had lower affinity for the NC-receptor. Dooley & Houghten (1996) reported that NC(1-13)NH$_2$ displaced [^{3}H]-NC as potently as NC or NCNH$_2$ in rat brain membranes while NC(1-12)NH$_2$ and NC(1-9)NH$_2$ were one and three orders of magnitude weaker than the natural peptide, respectively. We recently confirmed this order of affinity for NC fragments using mouse brain membranes (Varani *et al*., 1998). Butour *et al*. (1997) observed significant differences in K_i values between NC, NC(1-13), NC(1-12) and

NC(1-9) (0.13, 4.0, 145 and 4300 nM, respectively) in CHO_{NCR} cell membranes. However in this study [^{3}H]-NC and unamidated fragments were used. In our present studies with rat cerebrocortex and CHO_{NCR} we did not observe this large difference in potency between NC(1-12)NH_2 and NC(1-13)NH_2 in binding assays (but see below for cyclic AMP studies). These differences are difficult to explain but could potentially result from a number of factors including, the presence of peptidase inhibitors in our studies, differences in radioligand used, different buffer systems or a combination of these.

In functional studies, the ability to inhibit forskolin stimulated cyclic AMP accumulation was used as an indicator of NC-receptor activation in CHO_{NCR} cells. The use of cells transfected with a target receptor that couples with a particular cellular process enables investigation of drug interaction with the expressed receptor. $NCNH_2$, NC(1-13)NH_2 were as potent as NC, NC(1-12)NH_2 and [F/G]NC(1-13)NH_2 were 17 and 13 fold less potent respectively. NC(1-11)NH_2, NC(1-9)NH_2 were 200 fold and 1096 fold less potent than NC, respectively. IC_{50} of NC was similar to that previously reported (Fawzi *et al*., 1997; Adapa & Toll, 1997; Butour *et al*., 1997; Reinscheid *et al*., 1996). Butour *et al*. (1997) reported decreasing potency of NC by C-terminal truncation for inhibition of adenylyl cyclase (EC_{50} value of 0.8, 7.8, 67, >10,000 nM for NC, NC(1-13), NC(1-12) and NC(1-9) respectively) again using unamidated fragments. Reinscheid *et al*. (1996) reported the inability of NC(1-11) to inhibit adenylyl cyclase, claiming that the entire structure is necessary for biological activity. In the mouse vas deferens and the guinea-pig ileum, NC(1-13)NH_2 was the shortest fragment to retain activity similar to NC (Guerrini *et al*., 1997; Calo' *et al*., 1997) and is consistent with our data. Similar results were also obtained in electrically stimulated rat vas deferens (Calo' *et al*., 1998c) where NC(1-12)NH_2 was less potent (about 10 fold) than NC(1-13)NH_2. In addition, in the rat *in vivo* NC(1-13)NH_2 was as potent as NC in stimulating food intake while NC(1-12)NH_2 was inactive (Polidori *et al*., 1998).

In our hands [F/G]NC(1-13)NH_2 behaved as a full agonist in glutamate release and cyclic AMP studies. In a recent short report Butour *et al*. (1998) have also reported agonist action of [F/G]NC(1-13)NH_2 at the level of cyclic AMP accumulation in transfected CHO cells. This is in contrast to the original (Guerrini *et al*., 1998) inhibitory effects of this compound reported in mouse and guinea-pig peripheral tissues and with the data we obtained in the rat vas deferens (see below). Reduced cellular cyclic AMP levels produced by opioids inhibit the Ih current and is speculated to decrease action potential frequency (Ingram & Williams, 1994). Although inhibition of cyclic AMP is only one of the assumed mechanisms of action of NC following its binding to the NC-receptor, it provides valuable information on specific activation of the this receptor. The observed inhibition is PTX sensitive indicating the coupling to the G_i/G_o class of G-proteins. Consistent with these data we have shown that both NC and [F/G]NC(1-13)NH_2 actions on cyclic AMP formation are completely reversed by PTX pretreatment.

We have previously demonstrated that NC inhibits K^+ evoked glutamate release from rat cerebrocortical slices in a concentration-dependent and naloxone insensitive manner (Nicol *et al*., 1996). This assay system was used to further investigate the central effects of [F/G]NC(1-13)NH_2. [F/G]NC(1-13)NH_2 was as efficacious as $NCNH_2$ or NC for inhibition of K^+ evoked glutamate release. In addition, there was no difference between the inhibition produced by 300 nM [F/G]NC(1-13)NH_2, 300 nM $NCNH_2$ and 300 nM NC(1-13)NH_2. Agonist activity of [F/G]NC(1-13)NH_2 at central NC functional sites has also been described in other experimental models. In particular, [F/G]NC(1-13)NH_2 mimicked the action of NC (i) when injected intrathecally in the rat inducing analgesia (Xu *et al*., 1998; Candeletti *et al*., 1998), (ii) when injected intracerebroventricularly also in the rat inducing stimulation of food intake (Polidori *et al*., 1998); and finally (iii) when injected intracerebroventricularly in the mouse (Calo' *et al*., 1998b) inducing hyperalgesia and antimorphine actions. On the other hand, in the periphery [F/G]NC(1-13)NH_2 exhibits antagonist activity (Guerrini *et al*., 1998). Specifically, in the rat vas deferens, [F/G]NC(1-13)NH_2 antagonized NC effects with a pA_2 value of 6.76. Worthy of mention is the fact that the peptidase inhibitor cocktail increases the apparent affinity of NC (Nicholson *et al*; 1998b) but not that of the pseudopeptide, indicating (in line with the findings by Xu *et al*., 1998) that [F/G]NC(1-13)NH_2 is more metabolically stable compared with the natural peptide.

The early indications of [F/G]NC(1-13)NH_2 agonist activity at central NC functional sites and antagonist activity at peripheral NC functional sites is now not so well defined. Nicholson *et al*. (1998a) reported in rat cortex [^{35}S]GTPγS assay that [F/G]NC(1-13)NH_2 possessed some efficacy but also inhibited the response to NC with a pA_2 of 8.6. Similar results (intrinsic activity $\alpha = 0.45$, pA_2 7.2) were obtained by Schlicker *et al*. (1998) studying the inhibitory effect of NC on [^{3}H]-noradrenaline release from mouse cerebral cortex slices. Of particular interest to the present study is the report of Toll *et al*. (1998) in which [F/G]NC(1-13)NH_2 inhibited cyclic AMP formation and enhanced [^{35}S]-GTPγS binding in CHO and SH-SY5Y cells transfected with the human NC receptor. However, the authors reported that [F/G]NC(1-13)NH_2 was an antagonist at low levels of receptor expression and a partial agonist at high levels of expression.

What is the nature of the reported differences between central and peripheral NC functional sites? The most likely explanation is that [F/G]NC(1-13)NH_2 is a partial agonist at the NC receptor and that the strength of NC signaling is very high in the CNS and in high expressing transfected systems, and low in the periphery. If these suppositions are true, the estimated intrinsic activity of [F/G]NC(1-13)NH_2 will be near 0 in the periphery and near 1 in the CNS. An alternative but much more speculative explanation is that the differences may result from different NC receptor subtypes. However, the evidence in support of this hypothesis is scant and to date only a single gene encoding the NC receptor has been identified. Certainly, the currently identified splice variants show distribution patterns that encompass both central and peripheral tissues (Wang *et al*., 1994; Wick *et al*., 1995; Peluso *et al*., 1998). Addressing these questions will represent an important area for future studies.

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References

ABDULLA, F.A. & SMITH, P.A. (1997). Nociceptin inhibits T-type Ca^{2+} channel current in rat sensory neurons by a G-protein-independent mechanism. *J. Neurosci.,* **17,** 8721–8728.

ADAPA, I.D. & TOLL, L. (1997). Relationship between binding affinity and functional activity of nociceptin/orphanin FQ. *Neuropeptides,* **31,** 403–408.

ARDATI, A., HENNINGSEN, R.A., HIGELIN, J., REINSCHEID, R.K., CIVELLI, O. & MONSMA, Jr. F.J. (1997). Interaction of [^{3}H] orphanin FQ and ^{125}I-Tyr^{14}-orphanin FQ with the orphanin FQ receptor: kinetics and modulation by cations and guanine nucleotides. *Mol. Pharmacol.,* **51,** 816–824.

BUTOUR, J.L., MOISAND, C., MAZARGUIL, H., MOLLEREAU, C. & MEUNIER, J.C. (1997). Recognition and activation of the opioid receptor-like ORL1 receptor by nociceptin, nociceptin analogs and opioids. *Eur. J. Pharmacol.,* **321,** 97–103.

BUTOUR, J.L., MOISAND, C., MOLLEREAU, C. & MEUNIER, J.C. (1998). [Phe$^1\psi$(CH$_2$-NH)Gly^2]nociceptin-(1-13)-NH$_2$ is an agonist of the nociceptin (ORL1) receptor. *Eur. J. Pharmacol.,* **349,** R5–R6.

CALO', G., GUERRINI, R., BIGONI, R., RIZZI, A., BIANCHI, C., REGOLI, D. & SALVADORI, S. (1998a). Structure-Activity Study of the Nociceptin(1-13)-NH$_2$ N-Terminal Tetrapeptide and Discovery of a Nociceptin Receptor Antagonist. *J. Med. Chem.,* **41,** 3360–3366.

CALO', G., RIZZI, A., BODIN, M., NEUGEBAUER, W., SALVADORI, S., GEURRINI, R., BIANCHI, C. & REGOLI, D. (1997). Pharmacological characterization of nociceptin receptor. *Can. J. Physiol. Pharmacol.,* **75,** 713–718.

CALO', G., RIZZI, A., MARZOLA, G., GUERRINI, R., SALVADORI, S., BEANI, L., REGOLI, D. & BIANCHI, C. (1998b). Pharmacological characterization of the nociceptin receptor mediating hyperalgesia in the mouse tail withdrawal assay. *Br. J. Pharmacol.,* **125,** 373–378.

CALO', G., RIZZI, A., & REGOLI, D. (1998c). Pharmacology of nociceptin receptors. In *Peptidergic G-protein coupled receptors: from basic research to clinical applications*. eds Geppetti, P., Swartz, T.W., Regoli, D. & Muller-Esterl, W. Amsterdam, Netherlands: IOS press. (in press).

CANDELETTI, S., GUERRINI, R., CALO', G. & FERRI, S. (1998). Effects of the nociceptin receptor antagonist Phe$^1\psi$(CH$_2$NH)-Gly^{2}NC(1-13)NH$_2$ on nociception in rats. In *29th International Narcotic Research Conference*. **A170.** 20–25 July, 1998. Garmisch-Partenkirchen: Germany.

CONNOR, M., VAUGHAN, C.W., CHIENG, B. & CHRISTIE, M.J. (1996a). Nociceptin receptor coupling to a potassium conductance in rat locus coeruleus in vitro. *Br. J. Pharmacol.,* **119,** 1614–1618.

CONNOR, M., YEO, A & HENDERSON, G. (1996b). The effect of nociceptin on Ca^{2+} channel current and intracellular Ca^{2+} in the SH-SY5Y human neuroblastoma cell line. *Br. J. Pharmacol.,* **118,** 205–207.

DARLAND, T. & GRANDY, D.K. (1998). The orphanin FQ system: an emerging target for the management of pain? *Br. J. Anaesthesia.,* **81,** 29–37.

DOOLEY, C. & HOUGHTEN, R.A. (1996). Orphanin FQ: receptor binding and analog structure activity relationships in rat brain. *Life. Sci.,* **59,** 23–29.

FABER, E.S.L., CHAMBERS, J.P., EVANS, R.H. & HENDERSON, G. (1996). Depression of glutamatergic transmission by nociceptin in the neonatal rat hemisected spinal cord preparation in vitro. *Br. J. Pharmacol.,* **119,** 189–190.

FAWZI, A.B., ZHANG, H., WEIG, B., HAWES, B. & GRAZIANO, M.P. (1997). Nociceptin activation of the human ORL1 receptor expressed in Chinese hamster ovary cells: functional homology with opioid receptors. *Eur. J. Pharmacol.,* **336,** 233–242.

GIULIANI, S. & MAGGI, C.A. (1996). Inhibition of tachykinin release from peripheral endings of sensory nerves by nociceptin, a novel opioid peptide. *Br. J. Pharmacol.,* **118,** 1567–1569.

GUERRINI, R., CALO', G., RIZZI, A., BIANCHI, C., LAZARUS, L.H., SALVADORI, S., TEMUSSI, P.A. & REGOLI, D. (1997). Address and message sequences for the nociceptin receptor: A structure-activity study of nociceptin-(1-13)-peptide amide. *J. Med. Chem.,* **40,** 1789–1793.

GUERRINI, R., CALO', G., RIZZI, A., BIGONI, R., BIANCHI, C., SALVADORI, S. & REGOLI, D. (1998). A new selective antagonist of the nociceptin receptor. *Br. J. Pharmacol.,* **123,** 163–165.

HELYES, Z., NEMETH, J., PINTER, E. & SZOLCSANYI, J. (1997). Inhibition by nociceptin of neurogenic inflammation and the release of SP and CGRP from sensory nerve terminals. *Br. J. Pharmacol.,* **121,** 613–615.

INGRAM, S.L. & WILLIAMS, J.T. (1994). Opioid inhibition of Ih via adenylyl cyclase. *Neuron,* **13,** 179–186.

MATHIS, J.P., RYAN-MORO, J., CHANG, A., HOM, J.S.H., SCHEINBERG, D.A. & PASTERNAK, G.W. (1997). Biochemical evidence for orphanin FQ/nociceptin receptor heterogeneity in mouse brain. *Biochem. Biophys. Res. Commun.,* **230,** 462–465.

MATTHES, H., SEWARD, E.P., KIEFFER, B. & NORTH, R.A. (1996). Functional selectivity of orphanin FQ for its receptor coexpressed with potassium channel subunit in Xenopus laevis oocytes. *Mol. Pharmacol.,* **50,** 447–450.

MEUNIER, J.C. (1997). Nociceptin/orphanin FQ and the opioid receptor-like ORL1 receptor. *Eur. J. Pharmacol.,* **340,** 1–15.

MEUNIER, J.C., MOLLEREAU, C., TOLL, L., SUAUDEAU, C., MOISAND, C., ALVINERIE, P., BUTOUR, J.L., GUILLEMOT, J.C., FERRARA, P., MONSARRAT, B., MAZARGUIL, H., VASSART, G., PARMENTIER, M. & COSTENTIN, J. (1995). Isolation and structure of the endogenous agonist of opioid receptor-like ORL1 receptor. *Nature,* **377,** 532–535.

MURPHY, N.P., LY, H.T. & MAIDMENT, N.T. (1996). Intracerebroventricular orphanin FQ nociceptin suppresses dopamine release in the nucleus accumbens of anaesthetised rats. *Neuroscience,* **75,** 1–4.

NEAL, M.J., CUNNINGHAM, J.R., PATERSON, S.J. & McKNIGHT, A.T. (1997). Inhibition by nociceptin of the light-evoked release of Ach from retinal cholinergic neurones. *Br. J. Pharmacol.,* **120,** 1399–1400.

NICHOLSON, J.R., MASON, S.L., LEE, K. & McKNIGHT, A.T. (1998a). The effect of the agonist AC-RYYRWKNH$_2$ and the antagonist Phe$^1\psi$(CH$_2$NH)Gly^{2}NC(1-13)NH$_2$ at the ORL1 receptor of central and peripheral sites. In *29th International Narcotic Research Conference*. **A129.** 20-25 July, 1998. Garmisch-Partenkirchen: Germany.

NICHOLSON, J.R., PATERSON, S.J., MENZIES J.R.W., CORBETT, A.D. & McKNIGHT, A.T. (1998b). Pharmacological studies on the 'orphan' opioid receptor in central and peripheral sites. *Can. J. Physiol. Pharmacol.,* **76,** 304–313.

NICOL, B, LAMBERT, D.G., ROWBOTHAM, D.J., SMART, D. & McKNIGHT, A.T. (1996). Nociceptin induced inhibition of K^+ evoked glutamate release from rat cerebrocortical slices. *Br. J. Pharmacol.,* **119,** 1081–1083.

OKAWA, H., HIRST, R.A., SMART, D., McKNIGHT, A.T. & LAMBERT, D.G. (1998). Rat central ORL-1 receptor uncouples from adenylyl cyclase during membrane preparation. *Neurosci. Lett.,* **246,** 49–52.

PATEL, H.J., GIEMBYCZ, M.A., SPICUZZA, L., BARNES, P.J. & BELVISI, M.G. (1997). Naloxone-insensitive inhibition of acetylcholine release from parasympathetic nerves innervating guinea-pig trachea by the novel opioid, nociceptin. *Br. J. Pharmacol.,* **120,** 735–736.

PELUSO, J., LAFORGE, K.S., MATTHES, H.W., KREEK, M.J., KIEFFER, B.L. & GAV'ERIAUX-RUFF, C. (1998). Distribution of nociceptin/orphanin FQ receptor transcript in human central nervous system and immune cells. *J. Neuroimmunol.,* **81,** 184–192.

POLIDORI, C., CALO', G., GUERRINI, R., REGOLI, D. & MASSI, M. (1998). Pharmacological characterization of the nociceptin receptor mediating hyperphagia. 12th International Conference on the Physiology of Food and Fluid Intake (ICPFFI XII) Joint meeting with the Society for the Study of Ingestive Behaviour (SSIB). 5–8 July, 1998. Pecs: Hungary. pg. 31.

REINSCHEID, R.K., ARDATI, A., MONSMA, F.J. JR. & CIVELLI, O. (1996). Structure-activity relationship studies on the novel neuropeptide orphanin FQ. *J. Biol. Chem.,* **271,** 14163–14168.

REINSCHEID, R.K., NOTHACKER, H.P., BOURSON, A., ARDATI, A., HENNINGSEN, R.A., BUNZOW, J.R., GRANDY, D.K., LANGEN, H., MONSMA, F.J. JR & CIVELLI, O. (1995). Orphanin FQ: a neuropeptide that activates an opioidlike G protein-coupled receptor. *Science,* **270,** 792–794.

ROSSI, G.C., LEVENTHAL, L., BOLAN, E. & PASTERNAK, G.W. (1997). Pharmacological characterization of orphanin FQ/nociceptin and its fragments. *J. Pharmacol. Exp. Ther.,* **282,** 858–865.

SCHLICKER, E., WERTHWEIN, S., KATHMANN, M. & BAUER, U. (1998). Nociceptin inhibits noradrenaline release in the mouse brain cortex via presynaptic ORL1 receptors. *N.S. Arch. Pharmacol.,* **358,** 418–422.

TOLL, L., BURNSIDE, J. & BERZETEI-GURSKE, I. (1998). Agonist activity of ORL1 antagonists is dependent upon receptor number. In *29th International Narcotic Research Conference*. **A89.** 20–25 July, 1998. Garmisch-Partenkirchen: Germany.

VARANI, K., CALO, G., RIZZI, A., MERIGHI, S., TOTH, G., GUERRINI, R., SALVADORI, S., BOREA, P.A. & REGOLI, D. (1998). Nociceptin receptor binding in mouse forebrain membranes: thermodynamic characteristics and structure activity relationships. *Br. J. Pharmacol.,* **125,** 1485–1490.

VAUGHAN, C.W. & CHRISTIE, M.J. (1996). Increase by the ORL1 receptor (opioid receptor-like1) ligand, nociceptin, of inwardly rectifying K conductance in dorsal raphe nucleus neurones. *Br. J. Pharmacol.,* **117,** 1609–1611.

VAUGHAN, C.W., INGRAM, S.L. & CHRISTIE, M.J. (1997). Actions of the ORL1 receptor ligand nociceptin on membrane properties of rat periaqueductal gray neurons in vitro. *J. Neuroscience,* **17,** 996–1003.

WANG, J.B., JOHNSON, P.S., IMAI, Y., PERSICO, A.M., OZENBERGER, B.A., EPPLER, C.M. & UHL, G.R. (1994). cDNA cloning of an orphan opiate receptor gene family member and its splice variant. *FEBS. Lett.,* **348,** 75–79.

WICK, M.J., MINNERATH, S.R., ROY, S., RAMAKRISHNAN, S. & LOH, H.H. (1995). Expression of alternate forms of brain orphan receptor mRNA in activated human peripheral blood lymphocytes and lymphocytic cell lines. *Brain. Res. Mol. Brain. Res.,* **32,** 342–347.

XU, I.S., WIESANFELD-HALLIN, Z., & XU, X-J. (1998). [Phe$^1\psi$(CH$_2$-NH)Gly^2]-nociceptin-(1-13)NH$_2$, a proposed antagonist of the nociceptin receptor, is a potent and stable agonist in the rat spinal cord. *Neurosci. Lett.,* **249,** 127–130.

YABALURI, N. & MEDZIHRADSKY, F. (1997). Regulation of μ-opioid receptor in neural cells by extracellular sodium. *J. Neurochem.,* **68,** 1053–1061.

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