British Journal of Pharmacology (2002) 136, 1178–1184

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Direct and indirect inhibition by nociceptin/orphanin FQ on noradrenaline release from rodent cerebral cortex *in vitro*

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1 The modulation exerted by nociceptin/orphanin FQ (NC) on noradrenaline (NE) release in rodent cerebral cortex slices and synaptosomes was studied.

2 Rat, mouse and guinea-pig cortical slices and synaptosomes were preincubated with 0.1 μM [^{3}H]-NE and superfused. NE release was evoked by 2 min of electrical (3 Hz) stimulation in slices and by 1 min pulse of 10 mM KCl in synaptosomes.

3 In rat cortical slices, 0.01–3 μM NC reduced the evoked [^{3}H]-NE efflux ($E_{max}-54\%$), with a bell-shaped concentration-response curve, which regained its monotonic nature in the presence of either 0.1 μM naloxone (NX) or 30 μM bicuculline. In synaptosomes, the NC effect curve was sygmoidal in shape and reached a plateau at 1 μM concentration.

4 In the rat, both 1 μM [Phe$^1\psi$(CH$_2$-NH)Gly^2]NC(1-13)NH$_2$ and 10 μM [Nphe^1]NC(1-13)NH$_2$ (NPhe) antagonised NC-induced inhibition, without per se modifying [^{3}H]-NE efflux. The effects of 0.3–1 μM NC concentrations were partially prevented by 1 μM NX; 1 μM D-Phe-Cys-Thr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$ (CTOP) was also an effective antagonist, but 0.1 μM norbinaltorphimine was not.

5 In the mouse cerebral cortex, NC-induced inhibition of NE release (pEC_{50} 6.87, $E_{max}-61\%$, in the slices) was prevented by Nphe but was NX-insensitive. In guinea-pig cortical slices, NC effect (pEC_{50} 6.22, $E_{max}-38\%$) was prevented by Nphe, but was NX-insensitive.

6 These findings demonstrate that NC inhibits NE release from rodent cerebral cortex *via* presynaptically located ORL$_1$ receptors. In the rat, μ opioid and GABA$_A$ receptors are involved as well.

British Journal of Pharmacology (2002) **136,** 1178–1184

Keywords: Nociceptin/orphanin FQ; ORL$_1$ receptors; noradrenaline release; naloxone; (Nphe^1)nociceptin(1-13)NH$_2$; rodent cerebral cortex; slices; synaptosomes; GABA; species differences

Abbreviations: ANOVA, analysis of variance; Bic, bicuculline; CRC, concentration-response curve; CTOP, D-Phe-Cys-Thr-D-Trp-Orn-Thr-Pen-Thr-NH$_2$; GABA, γ-aminobutyric acid; NC, nociceptin/orphanin FQ; NE, noradrenaline; Nphe, [Nphe^1]nociceptin(1-13)NH$_2$; NX, naloxone; ORL$_1$, opioid receptor-like 1 receptor; St, electrical stimulation

Introduction

Since the discovery of the opioid receptor-like 1 (ORL$_1$) receptor (Mollereau *et al*., 1994; Bunzow *et al*., 1994) and its endogenous ligand nociceptin/orphanin FQ (NC) (Meunier *et al*., 1995; Reinscheid *et al*., 1995), a great effort has been made to assess its physiological role, and its involvement in pathological states, in order to predict pharmacological applications for both agonists and antagonists (for reviews see Henderson & McKnight, 1997; Meunier, 2000; Calò *et al*., 2000b; Mogil & Pasternak, 2001).

Despite the high degree of structural homology between the classical opioid receptors and ORL$_1$, which has been proposed as a fourth opioid receptor (Cox, 2000), neither opioid agonists nor antagonists display high affinity for ORL$_1$ and most NC actions are naloxone (NX)-insensitive (Henderson & McKnight, 1997). However, NX has been shown to effectively antagonize some of the NC effects, both *in vivo* (Rossi *et al*., 1996; Pomonis *et al*., 1996; Konya *et al*., 1998) and *in vitro* (Madamba *et al*., 1999).

In agreement with its transduction mechanism, namely inhibition of adenylylcyclase and of calcium entry as well as activation of a potassium conductance (Hawes *et al*., 2000; Harrison & Grandy, 2000), NC has been shown to directly inhibit the release of various neurotransmitters, both in the central nervous system and in periphery (Schlicker & Morari, 2000; Giuliani *et al*., 2000). In this context, the inhibitory effect of NC on 5-hydroxytryptamine release from rat cerebral cortex has recently been reported (Siniscalchi *et al*., 1999a; Sbrenna *et al*., 2000). AS far as NC modulation on noradrenaline (NE) release is concerned, two detailed studies, carried out in mouse brain slices (Schlicker *et al*., 1998; Werthwein *et al*., 1999) and a third study comparing *in vitro* and *in vivo* nociceptin-amide effects (Okawa *et al*., 2001) have been published. In the present work, these studies have been extended checking the existence of indirect NC effects involving other neurotransmitter systems. Moreover, the question of the presynaptic location of the receptors

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responsible for NC actions has been addressed by employing the synaptosomal preparation. Finally, since both quantitative and qualitative discrepancies in neuropeptide receptor binding and effects have been reported (Yoburn *et al*., 1991; Hall *et al*., 1993; Benyhe *et al*., 1999), we checked for possible differences between various rodent species – namely the rat, the mouse and the guinea-pig – as regards the inhibitory NC effect on NE release. Some of the present data have been preliminarily reported in abstract form (Siniscalchi *et al*., 1999b).

Methods

[^{3}H]-NE efflux

Vibratome-prepared fronto-parietal cortex slices (400 μm thick) from male Sprague-Dawley rats (200–300 g), Swiss mice (20–25 g) or guinea-pigs (350–400 g) were incubated at 37°C for 30 min in Krebs' solution (mM composition: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.25 (2.5 for the guinea-pig), KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10, ascorbic acid 0.05, disodium ethylenediaminetetracetate 0.03, continuously bubbled with 95% O_2, 5% CO_2) containing 0.1 μM [^{3}H]-NE (specific activity 14.56 Ci mmol^{-1}, DuPont NEN, Boston, MA, U.S.A.). The slices were then superfused at a flow rate of 0.25 ml min^{-1} with Krebs' solution in a thermoregulated (37°C) apparatus, made up of four 0.9 ml chambers, equipped with stimulating electrodes (Beani *et al*., 1978). Tritium overflow was evoked by electrical field stimulation (3 Hz, 30 mA cm^{-2}, 1 ms, for 2 min) at the 45th (St_1) and the 75th (St_2) min of superfusion. Superfusate samples were collected every 5 min from the 30th to the 95th min. The drugs under investigation were added to the medium from the 70th min onward, unless otherwise stated. At the end of the experiments, the radioactivity of the 5-min samples and of the slices (solubilized with 1 ml 1N NaOH) was determined by liquid scintillation counting; the fractional release, i.e. the amount of released tritium as a percentage of the tritium content at the onset of the respective collection period, was calculated for each sample. The net stimulation-evoked tritium efflux was calculated according to Beani *et al*. (1984): the expected basal efflux, assumed to decline linearly, was subtracted from the total amount released in the three samples (15 min) collected during and after stimulation. Drug effects were evaluated from the changes induced in the St_2/St_1 ratio, in comparison with control slices assayed in parallel. Synaptosomes were prepared from rat and mouse fronto-parietal cortex according to Sbrenna *et al*. (2000); the tissue was homogenized in 0.32 M sucrose buffered at pH 7.4 and centrifuged (10 min, $1000 \times g_{max}$, 4°C) to obtain a crude synaptosomal fraction, which was resuspended in Krebs' solution and centrifuged once more (20 min, $12{,}000 \times g_{max}$, 4°C). The pellet was diluted in 3 ml Krebs' solution and incubated with 0.1 μM [^{3}H]-NE at 33°C for 20 min. After incubation, aliquots of the suspension (~0.35 mg protein ml^{-1}) were slowly layered onto MSI nylon syringe filters (0.45 μm pore size) which were connected by tubing to a peristaltic pump. The filters were maintained in a thermoregulated (37°C) bath and superfused at a flow rate of 0.4 ml min^{-1}; the synaptosomes were stimulated by 10 mM KCl (1-min pulse) at the 21st min (Sbrenna *et al*., 2000). Drugs were added 9 min before KCl stimulation. At the end of the experiments, the radioactivity of the 3-min samples of perfusate and of the synaptosomes (solubilized with 1 ml 1N NaOH) was determined by liquid scintillation counting, and the fractional release was calculated as above.

Drugs

The peptides used in this study were prepared and purified as previously described (Guerrini *et al*., 1997; Calò *et al*., 1998). Naloxone, D-Phe-Cys-Thr-D-Trp-Orn-Thr-Pen-Thr-NH_2 and norbinaltorphimine were from Tocris Cookson (Bristol, U.K.); other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Stock solutions (1 mM) were made in distilled water and kept at −20°C until use.

Statistics

The data are expressed as mean ± s.e.mean of n experiments. Data have been statistically analysed by the Kruskal–Wallis analysis of the variance (ANOVA), or by two-way ANOVA, when appropriate; statistical significance of the differences has been assessed using the *post hoc* Dunn's multiple comparison test, *via* a software package (GraphPad Prism). P values lower than 0.05 were considered to be statistically significant. The pharmacological terminology adopted is in line with IUPHAR recommendations (Jenkinson *et al*., 1995).

Results

Spontaneous tritium efflux from control cerebral cortex slices at the 70th min of superfusion was 2.32 ± 0.18% of tissue tritium content in the rat (n = 32), 1.69 ± 0.10% in the mouse (n = 6), 2.01 ± 0.11% in the guinea-pig (n = 6) and was not modified by any tested drug. Electrical stimulation evoked a frequency-related, Na^+- and Ca^{2+}-dependent net tritium efflux, which may be considered an index of physiological NE release (data not shown, see also Schlicker *et al*., 1998). The [^{3}H]-NE efflux evoked by a 2-min 3 Hz stimulation was highest in the mouse, followed by the rat and the guinea-pig slices (Table 1). In control slices the St_2/St_1 ratios were always close to unity; NE release was not changed by any of the antagonists tested, added to the medium either throughout the superfusion, or at the 70th min (Tables 1 and 2).

In rat cerebral cortex slices, NC (0.01–3 μM) reduced 3 Hz-evoked [^{3}H]-NE efflux (Figure 1a); the concentration-response curve (CRC) was bell shaped, with the maximal effect (E_{max}, −54 ± 4% of the control) at 1 μM. The inhibition induced by NC was prevented by the ORL_1 antagonist (Nphe^1)nociceptin(1-13)NH_2 (Nphe), 10 μM, (Figure 1a); the effect of 1 μM NC was antagonized as well by the addition of 1 μM [Phe$^1\psi$(CH_2-NH)Gly^2]NC(1-13)NH_2 (St_2/St_1 0.82 ± 0.06, P < 0.01 *vs* NC alone, n = 7, Dunn's test).

In rat cerebral cortex synaptosomes, NC (3 nM–3 μM) did not modify basal tritium outflow but did inhibit, in a concentration dependent manner (pEC_{50} 7.82, Figure 1b), the [^{3}H]-NE efflux evoked by a 1-min pulse of 10 mM KCl; the CRC to NC was not bell-shaped and reached a plateau at 1 μM concentration; the NC effect was prevented by 10 μM Nphe (Figure 1b).

The different shape of the CRC to NC in the synaptosomal, *vs* slice, preparation raised the question of

Table 1 Electrically-evoked [^{3}H]-NE efflux from rodent cerebral cortex slices. Lack of effect of antagonists present in the medium throughout the superfusion

	Rat		*Mouse*		*Guinea-pig*	
Treatment (concentration)	*St_1-evoked [^{3}H]NE efflux (% of tissue tritium) [n]*	*St_2/St_1*	*St_1-evoked [^{3}H]NE efflux (% of tissue tritium) [n]*	*St_2/St_1*	*St_1-evoked [^{3}H]NE efflux (% of tissue tritium) [n]*	*St_2/St_1*
Control	3.04±0.20 [32]	0.94±0.03	3.67±0.59 [6]	0.89±0.09	2.30±0.55 [6]	0.90±0.03
Naloxone (1 μM)	2.85±0.36 [9]	0.84±0.07	3.29±0.66 [3]	0.91±0.21	2.35±0.50 [3]	0.89±0.08
D-Phe-Cys-Thr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) (1 μM)	2.73±0.18 [5]	0.89±0.10	–	–	–	–
Norbinaltorphimine (0.1 μM)	3.15±0.42 [5]	1.07±0.07	–	–	–	–
[Nphe^1]nociceptin(1-13)NH_2 (10 μM)	2.63±0.37 [6]	0.97±0.10	4.02±0.98 [3]	0.87±0.08	2.46±0.53 [6]	0.90±0.03
[Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin (1-13)NH_2 (1 μM)	3.16±0.44 [3]	1.08±0.07	–	–	–	–
Bicuculline (30 μM)	2.61±0.31 [4]	0.86±0.02	–	–	–	–

Slices were electrically stimulated (3 Hz, for 2 min) at the 45th and 75th min of superfusion. Two-way analysis of variance assessed a significant ($P<0.05$) species difference.

Table 2 Electrically-evoked [^{3}H]-NE efflux from rodent cerebral cortex slices. Lack of effect of antagonists added to the medium at the 70th min of superfusion

Treatment (concentration)	*Rat*		*Mouse*		*Guinea-pig*	
Treatment (concentration)	*St_2/St_1*	*[n]*	*St_2/St_1*	*[n]*	*St_2/St_1*	*[n]*
Naloxone (1 μM)	1.00±0.10	[7]	1.09±0.12	[3]	0.96±0.08	[3]
D-Phe-Cys-Thr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) (1 μM)	0.87±0.09	[3]	–		–	
Norbinaltorphimine (0.1 μM)	1.07±0.18	[3]	–		–	
[Nphe^1]NC(1-13)NH_2 (10 μM)	0.97±0.05	[4]	1.00±0.20	[3]	0.99±0.12	[3]
[Phe$^1\psi$(CH_2-NH)Gly^2]NC(1-13)NH_2 (1 μM)	0.99±0.05	[8]	–		–	
Bicuculline (30 μM)	0.87±0.13	[3]	–		–	

Slices were electrically stimulated (3 Hz, for 2 min) at the 45th and 75th min of superfusion.

an indirect action responsible for the reduced effect displayed by 3 μM NC in the latter preparation. Thus, experiments were carried out in the slices to check the involvement of other systems, namely opioid peptides and GABA. As shown in Figure 2, in the presence of either the opioid antagonist NX at a low concentration (0.1 μM), or of the $GABA_A$ antagonist bicuculline (30 μM) the inhibition induced by 0.3 and 1 μM NC remained unchanged, while at 3 μM NC it deepened, unveiling a linear concentration/effect relationship.

A higher concentration (1 μM) of the opioid antagonist did not counteract the inhibition induced by 0.1 μM NC but, unexpectedly, was able to significantly prevent the effects of 0.3 and 1 μM NC, both in slices (Figure 3a) and in synaptosomes (Figure 3b). The inhibitory effect induced in slices by 1 μM NC concentration, however, was not completely antagonized by NX, even at a higher concentration (1 μM NC plus 3 μM NX = 79±11%, $n=9$, $P<0.05$ *vs* 1 μM NC and *vs* the respective control, Dunn's test). Similarly, Nphe only partially prevented the effect of 1 μM NC when added alone at 10 μM (see Figure 1) or 30 μM concentration (1 μM NC plus 30 μM Nphe = 83±9%, $n=3$, $P<0.05$ *vs* 1 μM NC and *vs* the respective control, Dunn's test). Only when both 10 μM Nphe and 1 μM NX were added to the superfusion medium together could they fully antagonize the 1 μM NC effect (Figure 3).

To rule out any possible effect the investigated drugs might have on NE uptake mechanisms, further experiments were carried out in rat cortical slices superfused in the presence of the selective NE uptake inhibitor, desipramine, 1 μM. Under the latter experimental conditions, the inhibitory effect induced by NC on 3 Hz-evoked [^{3}H]-NE efflux was confirmed: St_2/St_1 ratio was reduced to 54±6% of the controls by 1 μM NC ($P<0.05$, Dunn's test, $n=4$) and to 67±6% by 3 μM NC ($P<0.05$, $n=8$). The sensitivity to NX of NC effect was confirmed as well: in the presence of 1 μM NC plus 1 μM NX the St_2/St_1 ratio was 82±9% of the controls ($P<0.05$ *vs* 1 μM NC alone, $n=4$).

In order to check for the type of opioid receptors involved in the NC effect, the selective μ antagonist D-Phe-Cys-Thr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) was tested: at 1 μM concentration CTOP significantly reduced NC inhibition, while the selective κ opioid receptor antagonist, norbinaltorphimine, 0.1 μM, did not (Table 3).

In mouse cerebral cortex slices, NC inhibited 3 Hz-evoked [^{3}H]-NE efflux with an E_{max} of −61±5% and a pEC_{50} value of 6.87. The effect of 0.3 μM NC was antagonized by 10 μM Nphe, but not by 1 μM NX (Figure 4). In the same way, in mouse cerebral cortex synaptosomes, NC (0.3 μM) inhibited [^{3}H]-NE efflux evoked by a 1-min pulse of 10 mM KCl; the effect was antagonized by 10 μM Nphe, but not by 1 μM NX (Figure 4).

In guinea-pig cerebral cortex slices, NC inhibited 3 Hz-evoked [^{3}H]-NE efflux with a E_{max} of −38±12% at 3 μM,

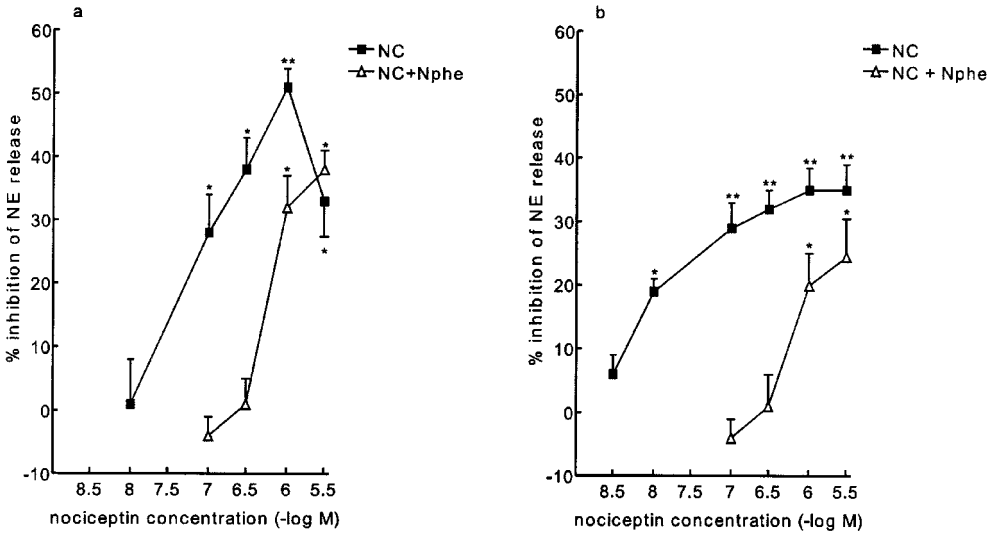

Figure 1 (a) Electrically (3 Hz)-evoked tritium efflux from rat cerebral cortex slices preincubated with [^{3}H]-NE and stimulated for 2 min at the 45th (St_1) and 75th min (St_2) of superfusion: concentration-response curve (CRC) to nociceptin/orphanin FQ (NC), in the absence and in the presence of 10 μM [Nphe^1]nociceptin(1-13)NH_2 (Nphe). Abscissa: NC molar concentration (logarithmic scale); ordinate: per cent inhibition of St_2/St_1 ratio. (b) [^{3}H]-NE overflow evoked by 10 mM KCl from rat cerebral cortex synaptosomes: CRC to NC in the absence and in the presence of 10 μM Nphe. Abscissa: NC molar concentration (logarithmic scale); ordinate: per cent inhibition of NE release. Points represent means$\pm$s.e.mean of 5–15 experiments. *$P<0.05$; **$P<0.01$, significantly different from the corresponding control, Kruskal–Wallis analysis of variance (ANOVA), followed by Dunn's multiple comparison test. A two-way ANOVA assessed a significant ($P<0.05$) difference between groups in the absence and in the presence of Nphe.

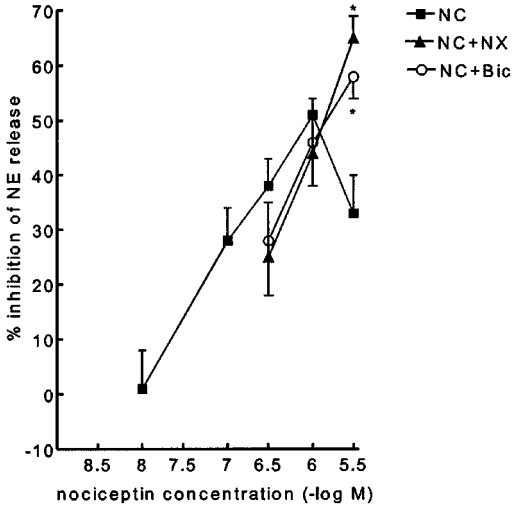

Figure 2 Electrically (3 Hz)-evoked tritium efflux from rat cerebral cortex slices preincubated with [^{3}H]-NE and stimulated for 2 min at the 45th (St_1) and 75th min (St_2) of superfusion: concentration-response curve to nociceptin/orphanin FQ (NC), in the absence and in the presence of either 0.1 μM naloxone (NX) or 30 μM bicuculline (Bic). Abscissa: NC molar concentration (logarithmic scale); ordinate: per cent inhibition of St_2/St_1 ratio. Points represent means$\pm$s.e.mean of 5–15 experiments. *$P<0.05$; **$P<0.01$, significantly different from NC alone, Kruskal–Wallis analysis of variance, followed by Dunn's multiple comparison test.

and a pEC_{50} value of 6.22. The effect of 0.3 μM NC (-16$\pm$7% of the controls, $n=6$, significantly less than in the other rodent species, $P<0.05$, Dunn's test) was antagonized by 10 μM Nphe (St_2/St_1 0.90$\pm$0.03, $n=6$, $P<0.001$ *vs* NC 0.3 μM alone), but not by 1 μM NX (St_2/St_1 0.71$\pm$0.09, $n=4$, $P<0.05$ *vs* controls).

Discussion

The present study reports: (a) a clear NC-induced inhibition of electrically-evoked NE release from the cerebral cortex of all three rodent species and, for the first time: (b) the presynaptic location of the receptors responsible for NC action; (c) the involvement of opioid and GABA systems in NC effects in the rat; and (d) the existence of differences in antagonist sensitivity among the three species studied.

NC inhibition of cortical NE release via *ORL_1 receptors*

In all the rodent species studied, NC displayed a significant inhibition of NE release and, in agreement with previous reports (Schlicker *et al*., 1998; Werthwein *et al*., 1999), a rank order sensitivity (mouse > rat > guinea-pig) to the NC effect was found. In all the rodent species, the involvement of ORL_1 receptors was demonstrated by the effectiveness of the selective antagonist Nphe in preventing NC action. Moreover, in this, as in other experimental models (Calò *et al*., 2000a) the latter compound was devoid of intrinsic activity, thus proving its usefulness as a pharmacological tool in studying ORL_1 receptor-mediated effects. Additional experiments were carried out in the rat with [Phe$^1\psi$(CH_2-NH_2)Gly^2]NC(1-13)NH_2, which has proved selective for ORL_1 receptors (Guerrini *et al*., 1998). In the present work, partially at variance with previous reports (Schlicker *et al*., 1998; Siniscalchi *et al*., 1999a), this compound behaved as a pure antagonist, preventing NC effect when added to the superfusion medium throughout the entire experiment, without displaying any residual agonist activity. Note that the antagonists, *per se*, proved ineffective in modifying cortical NE release (see Tables 1 and 2) thus ruling out the existence not only of a GABAergic but also of an endogenous opioid-

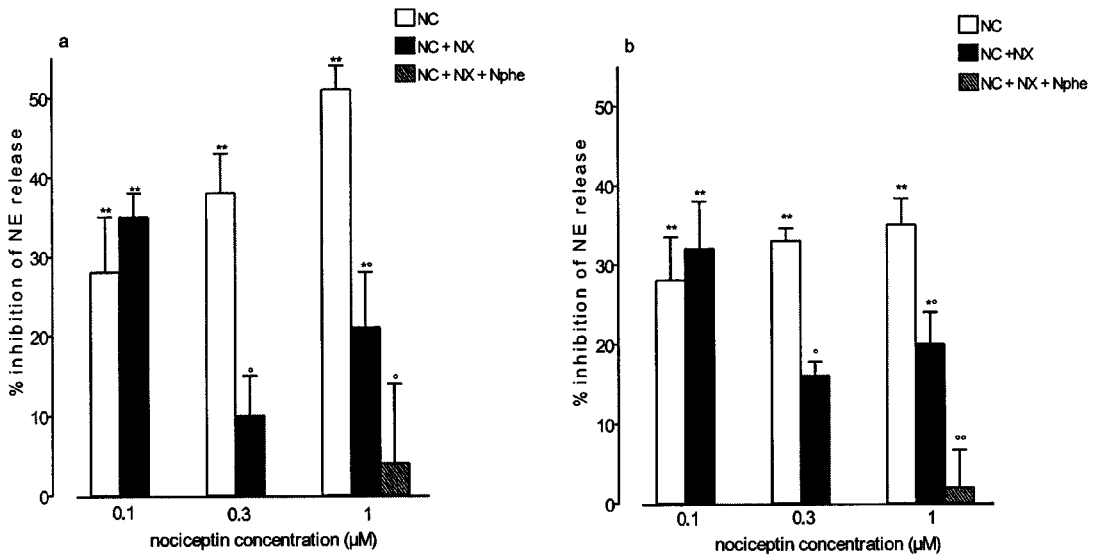

Figure 3 (a) Rat cerebral cortex slices preincubated with [^{3}H]-NE and electrically (3 Hz) stimulated for 2 min at the 45th (St_1) and 75th min (St_2) of superfusion. Effects of different concentrations of nociceptin/orphanin FQ (NC) and antagonism by 1 μM naloxone (NX), alone or together with 10 μM [Nphe^1]nociceptin(1–13)NH_2 (Nphe). (b) Rat cerebral cortex synaptosomes stimulated with a 1-min pulse of 10 mM KCl. Effects of different NC concentrations and antagonism by 1 μM NX, alone or together with 10 μM Nphe. Data are means ± s.e.mean of at least five experiments. $^*P<0.05$; $^{**}P<0.01$, significantly different from the corresponding control; $^\circ P<0.05$, $^{\circ\circ}P<0.01$, significantly different from NC alone, Kruskal–Wallis analysis of variance, followed by Dunn's multiple comparison test.

Table 3 Electrically-evoked [^{3}H]-NE efflux from rat cerebral cortex slices. Effect of antagonists on nociceptin-induced inhibition.

Treatment through-out the superfusion	*Treatment at the 70th min*	*St_2/St_1*	*[n]*	*% of the respective controls*
–	Nociceptin 1 μM	0.46 ± 0.03*	*[29]*	49 ± 3
D-Phe-Cys-Thr-D-Trp-Orn-Thr-Pen-Thr-NH_2 (CTOP) 1 μM	Nociceptin 1 μM	0.61 ± 0.05†	*[8]*	67 ± 8
Norbinaltorphimine 0.1 μM	Nociceptin 1 μM	0.53 ± 0.08*	*[6]*	52 ± 8

Slices were electrically stimulated (3 Hz, for 2 min) at the 45th and 75th min of superfusion. $^*P<0.01$, significantly different from the respective controls (antagonists present in the medium throughout the whole experiment, see Table 1), †$P<0.05$, significantly different from NC 1 μM alone, Kruskal–Wallis analysis of variance, followed by Dunn's multiple comparison text.

and an ORL_1-mediated modulatory tone (Fiber & Etgen, 1997; Schoffelmeer *et al*., 1991; Schlicker & Morari, 2000; Okawa *et al*., 2001). Clearly, the outflow of these transmitters in the slices was not relevant, due to the absence of GABA membrane carrier inhibitors and the relatively low frequency of stimulation (Raiteri *et al*., 1989; Bartfai *et al*., 1988).

Presynaptic location of ORL_1 receptors

Both the NC inhibitory effect on stimulus-evoked NE release and the antagonism by Nphe are present not only in the cortical slices, but also in the synaptosomes, suggesting that the receptors responsible for these actions are located presynaptically on the axon terminals of cortical noradrenergic neurons (see Schlicker & Morari, 2000).

Involvement of opioid and GABA systems in NC action in the rat

Another important finding is that, in agreement with previous observations in various experimental models (Jenck *et al*., 1997; Morikawa *et al*., 1998; Siniscalchi *et al*., 1999a), a bell-shaped CRC to NC was obtained in the rat cortical slice preparation. This finding might be attributed either to ORL_1 receptor desensitisation induced by the high NC concentration (see Werthwein *et al*., 1999; Siniscalchi *et al*., 1999a, for a detailed discussion), or to the presence of two subpopulations of binding sites with different affinity for the peptide and differently coupling to adenylylcyclase (Onali *et al*., 2001). However, the sygmoidal shape of the curve obtained in synaptosomes contradicts these explanations. Therefore, we tested the hypothesis that the lower inhibition shown by 3 μM NC in slices involved other neurotransmitters. Interestingly, in the presence of either NX, 0.1 μM, or bicuculline, 30 μM, the effects of 0.3 and 1 μM NC did not significantly change, but the inhibition by 3 μM NC deepened, so that the relationship between NC concentrations and NE inhibition regained its monotonic nature (see Figure 2). A possible explanation for these findings could be an inhibition, induced by the high NC concentration, on the release of opioid peptides (Gintzler *et al*., 1997) which, in turn, inhibit GABA (Vaughan *et al*., 1997); the latter has been reported to facilitate NE release (Fiber & Etgen, 1997; 1998; Fassio *et al*., 1999). The resulting effect would be a reduced inhibition of NE release by NC. Interestingly, a disinhibitory, bicuculline-sensitive, NC effect on the firing rate of thalamic neurons has

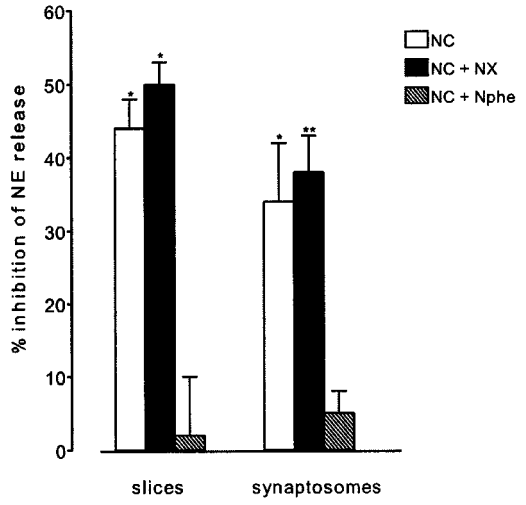

Figure 4 Mouse cerebral cortex slices and synaptosomes, preincubated with [^{3}H]-NE. Slices were electrically (3 Hz) stimulated for 2 min at the 45th (St_1) and 75th min (St_2) of superfusion; synaptosomes were stimulated with a 1-min pulse of 10 mM KCl. Effects of 1 μM naloxone (NX) and 10 μM [Nphe^1]NC(1-13)NH_2 (Nphe) on the inhibition induced by 0.3 μM nociceptin/orphanin FQ (NC) on the evoked tritium efflux. Data are means$\pm$s.e.mean of at least five experiments. *$P<0.05$; **$P<0.01$, significantly different from the corresponding control, Kruskal–Wallis analysis of variance, followed by Dunn's multiple comparison test.

been reported *in vivo* (Albrecht *et al*., 2001). Obviously, this hypothesis needs to be tested through further experimental study carried out with the model of cortical slices.

When NX was used at a higher concentration (1 μM), NC-induced inhibition of NE release was partially prevented both in slices, and in synaptosomes (see Figure 3). Probably, at a low (0.1 μM) concentration, NC directly inhibits NE release *via* ORL_1 receptors (antagonized by Nphe and not by NX) whereas at higher NC concentrations (0.3–1 μM) opioid receptors come into play (direct activation? receptor/receptor interaction? see Levac *et al*., 2002). Note that the possibility of interference by NE reuptake mechanisms on the observed effects was ruled out by the agreement between the results obtained in the presence and in the absence of desipramine.

To investigate which opioid receptor subtype was involved in the NC effect, other opioid ligands, more selective than NX, were tested. The κ antagonist norbinaltorphimine was ineffective, thus ruling out any role for κ receptors. This result is partially at variance with Madamba *et al*. (1999), who reported a NX- and norbinaltorphimine-sensitive increase in K^+ currents evoked by NC in rat hippocampal neurones, but the different brain area investigated may explain the difference. Instead, the partial effectiveness of the selective antagonist CTOP in antagonizing NC inhibition stands in favour of the involvement of μ receptors in the rat cerebral cortex.

Species differences in NC action

Indeed, one of the most interesting findings of the present work was the species differences in NC-induced inhibition of NE release: such differences were both quantitative (see above) and qualitative. In fact, the possible co-operation of μ opioid receptors in NC-induced inhibition of NE release, suggested by the partial sensitivity to NX and CTOP, was only displayed in the cerebral cortex of the rat. Therefore, caution is required in extrapolating experimental data from one animal species to another, and even more so in extending data obtained in rodents to humans (Bigoni *et al*., 2001).

The Authors wish to thank Dr Remo Guerrini, who synthesized the peptides used in this study, and Dr Girolamo Calò, for the helpful discussion of the results. The Authors also wish to thank Dr Eileen N. Cartoon for linguistic revision of this manuscript. This work was supported by CNR grant 9704432CT04 and Cofin MURST 1999.

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(Received April 8, 2002
Revised May 29, 2002
Accepted June 13, 2002)