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Inhibitory effect of nociceptin on [^{3}H]-5-HT release from rat cerebral cortex slices

[1]Anna Siniscalchi, [1]Donata Rodi, [1]Lorenzo Beani & *,[1]Clementina Bianchi

[1]Department of Clinical and Experimental Medicine, Section of Pharmacology, University of Ferrara, 44100 Ferrara, Italy

1 The effect of nociceptin (NC) on 5-hydroxytryptamine (5-HT) release was studied in rat cerebral cortex slices preincubated with [^{3}H]-5-HT and electrically stimulated (3 Hz, for 2 min) at the 45th (St_1) and the 75th (St_2) min of superfusion.

2 NC (0.1–3 μM), present in the medium from the 70th min onward, concentration-dependently reduced electrically evoked [^{3}H]-5-HT efflux ($pEC_{50} = 6.54$, E_{max} −54%). The inhibition was not antagonized by naloxone (1 μM) ruling out the involvement of opioid receptors.

3 Phe$^1\psi$(CH$_2$-NH$_2$)Gly^2]NC(1-13)NH$_2$, which acts as an opioid-like receptor (ORL_1) antagonist at the peripheral level, behaved as a partial agonist in cerebral cortex slices i.e. it inhibited [^{3}H]-5-HT efflux when added before St_2, however, when present in the medium throughout the whole experiment, [Phe$^1\psi$(CH$_2$-NH$_2$)Gly^2]NC(1-13)NH$_2$ prevented the action of NC added at the 70th min.

4 The non-selective ORL_1 receptor antagonist, naloxone benzoylhydrazone (3 μM), in the presence of 10 μM naloxone, did not modify the St_2/St_1 ratio but completely abolished the NC effect.

5 These findings demonstrate that NC inhibits 5-HT release from rat cerebral cortex slices *via* ORL_1 receptors, suggesting its involvement in central processes mediated by 5-HT.

Keywords: Nociceptin; ORL_1 receptors; 5-HT release; cerebral cortex; rat

Abbreviations: EDTA, disodium ethylenediaminetetracetate; [F/G]NC(1-13)NH$_2$, [Phe$^1\psi$(CH$_2$-NH$_2$)Gly^2]NC(1-13)NH$_2$; 5-HT, 5-hydroxytryptamine; NBH, naloxone benzoylhydrazone; NC, nociceptin; NX, naloxone; ORL_1, opioid-like receptor; St, electrical stimulation; TTX, tetrodotoxin

Introduction

The widespread distribution and localization of the opioid-like receptor (ORL_1) in the brain suggests that it may modulate a variety of central processes (Meunier, 1997; Henderson & McKnight, 1997; Darland & Grandy, 1998), including synaptic transmission. Both direct and indirect evidence suggests that the endogenous ORL_1 receptor ligand, nociceptin/orphanin FQ (NC) (Meunier *et al*., 1995; Reinscheid *et al*., 1995), inhibits neurotransmitter release (Nicol *et al*., 1996; Vaughan *et al*., 1997; Schlicker *et al*., 1998; Werthwein *et al*., 1999).

The importance of 5-hydroxytryptamine (5-HT) in several pathophysiological conditions has long been known (Bradley *et al*., 1992). In particular, 5-HT has been implicated in pain perception (Richardson, 1992) and anxiety (Hamon, 1994), conditions in which a role for NC has also been proposed (Meunier *et al*., 1995; Reinscheid *et al*., 1995; Jenck *et al*., 1997). Since high levels of ORL_1 have been demonstrated in the rat cerebral cortex (Bunzow *et al*., 1994) the effect of NC on 5-HT release in this brain area has been examined.

To date, no selective antagonist for the ORL_1 receptor has been described, except [Phe$^1\psi$(CH$_2$-NH$_2$)Gly^2]NC(1-13)NH$_2$ ([F/G]NC(1-13)NH$_2$) (Guerrini *et al*., 1998), which competitively antagonizes NC mediated inhibition of the electrically induced contractions of the guinea-pig ileum and mouse vas deferens. In the present study, [F/G]NC(1-13)NH$_2$, the opioid receptor antagonist naloxone and the non-selective opioid/ORL_1 competitive antagonist naloxone benzoylhydrazone (Nicholson *et al*., 1998; Schlicker *et al*., 1998) were used in order to characterize pharmacologically the effects of NC on [^{3}H]-5-HT release from rat cerebral cortex slices. A preliminary report of this work has been presented to the British Pharmacological Society (Siniscalchi *et al*., 1999).

*Author for correspondence at: Department of Clinical and Experimental Medicine, Section of Pharmacology, via Fossato di Mortara 17-19, 44100 Ferrara, Italy; E-mail: bnc@ifeuniv.unife.it

Methods

[^{3}H]-5-HT efflux

Vibratome-prepared fronto-parietal cortex slices (400 μm thick) from male Sprague-Dawley rats (200–300 g) were incubated, at 37°C for 30 min, in Krebs' solution (mM composition: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 10, ascorbic acid 0.05, disodium ethylenediaminetetracetate (EDTA) 0.03, continuously bubbled with 95% O_2, 5% CO_2) containing 50 nM [^{3}H]-5-HT (specific activity 27.8 Ci mmol^{-1}, Du Pont NEN, Boston, MA, U.S.A.). The slices were then superfused at a flow rate of 0.25 ml min^{-1} with Krebs' solution containing 3 μM paroxetine, in a thermoregulated (37°C) apparatus, constituted of four chambers (0.5 ml volume) equipped with stimulating electrodes (Beani *et al*., 1978). Tritium overflow was evoked by electrical field stimulation (1–10 Hz, 100 mA cm^{-2}, 2 msec, 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. In one group of experiments, slices were submitted to four stimulation periods, at the 45th, 65th, 85th and 105th min of superfusion, and 5 min samples were collected from the 35th to the 120th min. At the end of the experiments, the radioactivity of the 5 min samples and of the slices (solubilized in 1 ml 1 N NaOH) was determined by liquid scintillation counting, and 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 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. In the experiments with four stimulation periods the St_3/St_1 and the St_4/St_1 ratios were also calculated and compared.

Drugs

The peptides used in this study were prepared and purified as previously described (Guerrini *et al.*, 1997; Calò *et al.*, 1998). Naloxone was from Tocris Cookson (Bristol, U.K.). Other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or E. Merck (Darmstadt, Germany). Stock solutions (1 mM) were made in distilled water and kept at $-20^{\circ}C$ until use.

Statistics

The data are expressed as mean $\pm$ s.e.mean of n experiments. Data have been statistically analysed by analysis of the variance, followed by calculation of regression lines, when appropriate; statistical significance of differences has been assessed by the Mann-Whitney test, using a software package (Tallarida & Murray, 1987). P values lower than 0.05 were considered to be statistically significant. The pharmacological terminology adopted is in line with recent IUPHAR recommendations (Jenkinson *et al.*, 1995).

Results

In control slices, spontaneous tritium efflux at the 65th min was $4.31 \pm 0.16\%$ of tissue tritium and was not modified by any tested drugs. Electrical stimulation evoked a frequency-related, net [^{3}H]-5-HT efflux which was virtually abolished either by tetrodotoxin (TTX) 0.5 μM addition, or by Ca^{2+} ion omission from the 60th min of superfusion onward (Figure 1). In control slices the St_2/St_1 ratios were always close to 1. For routine experiments, a 3 Hz frequency of stimulation was chosen, since it is close to the physiological firing rate of 5-HT neurons *in vivo* (see Wilkinson & Middlemiss, 1992).

NC (0.1 – 3 μM) concentration-dependently reduced [^{3}H]-5-HT efflux evoked by 3 Hz stimulation (Figure 2). The effect of NC was statistically significant at 0.1 μM, and inhibition was maximal (by $54 \pm 5\%$) at 1 μM; the highest NC concentration tested (3 μM) did not induce a greater inhibition; instead, a bell-shaped concentration response curve was noted. The apparent NC pEC_{50}, graphically determined from the linear part of the curve (see Schlicker *et al.*, 1998) was 6.54. One group of experiments was carried out in order to test the occurrence of receptor desensitization. Slices were exposed to four stimulations, at the 45th, 65th, 85th and 105th min of superfusion. NC (1 μM), present in the medium from the 60th min onward, reduced St_2/St_1 to $53 \pm 1\%$, St_3/St_1 to $52 \pm 9\%$, St_4/St_1 to $47 \pm 7\%$ ($P < 0.05$ vs the corresponding controls, $n = 4$). Naloxone (NX, 1 μM) neither modified *per se* the stimulation-induced [^{3}H]-5-HT efflux when added at the 70th min (St_2/St_1 1.07 ± 0.10, $n = 4$) nor prevented the inhibitory effect of NC, when present in the medium from the onset of superfusion (Table 1).

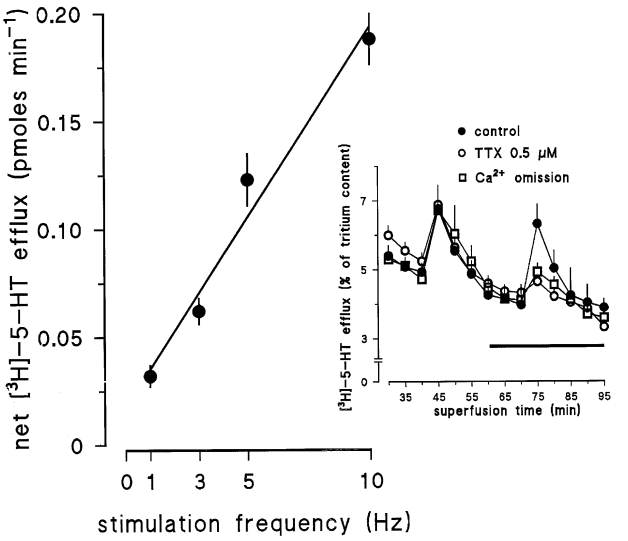

Figure 1 Frequency-dependence of electrically-evoked tritium efflux from rat cerebral cortex slices, preincubated with [^{3}H]-5-HT and stimulated for 2 min at the 45th (St_1) and 75th min (St_2) of superfusion. Abscissa: frequency of stimulation (Hz); ordinate: net extra efflux of [^{3}H]-5-HT evoked by St_1 (pmoles min^{-1}). Inset: Na^+- and Ca^{2+}-dependence of 3Hz-stimulation evoked [^{3}H]-5-HT efflux. The horizontal bar indicates either the presence of 0.5 μM tetrodotoxin (TTX), or the absence of Ca^{2+} ions in the superfusion medium. Abscissa: time of superfusion; ordinate: fractional tritium efflux (per cent of tritium content). Points represent means $\pm$ s.e.mean of five experiments.

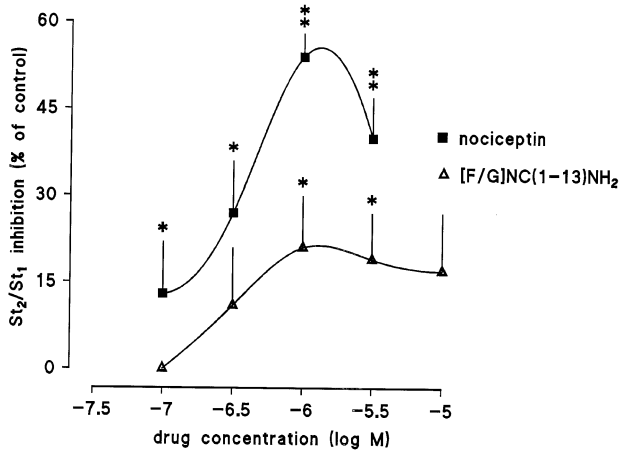

Figure 2 Concentration response curves to nociceptin (NC) and to [F/G]NC(1-13)NH_2 on electrically (3 Hz)-evoked [^{3}H]-5-HT efflux from rat cerebral cortex slices. Abscissa: molar drug concentration (logarithmic scale); ordinate: per cent inhibition of St_2/St_1 ratio. Points represent means $\pm$ s.e.mean of five experiments. $^*P < 0.05$; $^{**}P < 0.01$; significantly different from the corresponding control, Mann-Whitney U-test.

[F/G]NC(1-13)NH_2, added to the medium at the 70th min, significantly inhibited stimulation-induced [^{3}H]-5-HT efflux (Figure 2), although with a lower efficacy than NC (E_{max} $21 \pm 9\%$ inhibition, $P < 0.01$ vs NC E_{max}). When both 1 μM NC and 1 μM [F/G]NC(1-13)NH_2 were added together to the superfusion medium from the 70th min onward, the St_2/St_1 ratio was reduced to 0.72 ± 0.06 ($n = 4$, $P < 0.05$ vs the control). The degree of inhibition (by 29%) was lower ($P < 0.05$) than that induced by 1 μM NC alone (by 54%) and only slightly higher than that induced by 1 μM [F/G]NC(1-13)NH_2 alone (by 21%). In contrast, the effect of NC was prevented by [F/G]NC(1-13)NH_2 (1 μM), when superfused throughout the

Table 1 Effect of antagonists on the nociceptin-induced inhibition of [^{3}H]-5-HT efflux from rat cerebral cortex slices

Drugs added to the medium (μM) *Throughout the superfusion*	*At the 70th min*	*St_1-evoked [^{3}H]-5-HT efflux* (% of tissue tritium)	St_2/St_1	n
–	–	2.53±0.34	1.05±0.05	23
–	Nociceptin 1	3.02±0.43	0.51±0.04[a]	9
Naloxone 1	–	2.48±0.40	1.02±0.06	4
Naloxone 1	Nociceptin 1	3.03±0.52	0.54±0.16[b]	5
[F/G]NC(1-13)NH_2 1	–	2.32±0.52	1.03±0.08	6
[F/G]NC(1-13)NH_2 1	Nociceptin 1	2.15±0.66	1.03±0.09[c]	4
Naloxone 10+naloxone benzoylhydrazone 3	–	2.82±0.36	0.93±0.10	4
Naloxone 10+naloxone benzoylhydrazone 3	Nociceptin 1	2.85±0.03	1.05±0.11[c]	8

Slices were electrically stimulated (3 Hz, for 2 min) at the 45th and 75th min of superfusion. [a]Significantly different from the corresponding control, $P<0.025$, Mann-Whitney U-test; [b]Significantly different from naloxone 1 μM, $P<0.01$, Mann-Whitney U-test; [c]Significantly different from the group with NC 1 μM at the 70th min, $P<0.01$, Mann-Whitney U-test.

Table 2 Effect of peptidase inhibitors on the nociceptin-induced inhibition of [^{3}H]-5-HT efflux from rat cerebral cortex slices

Drugs added to the medium (μM) *Throughout the superfusion*	*At the 70th min*	*St_1-evoked [^{3}H]-5-HT efflux* (% of tissue tritium)	St_2/St_1	n
–	–	2.53±0.34	1.05±0.05	23
–	Nociceptin 0.1	2.37±0.47	0.81±0.13[a]	6
–	Nociceptin 0.3	2.18±0.36	0.77±0.10[b]	10
Amastatin 30, bestatin 30, captopril 30, phosphoramidon 30	–	2.49±0.26	1.06±0.10	6
Amastatin 30, bestatin 30, captopril 30, phosphoramidon 30	Nociceptin 0.1	2.29±0.30	0.82±0.09[a]	5
Amastatin 30, bestatin 30, captopril 30, phosphoramidon 30	Nociceptin 0.3	2.28±0.31	0.73±0.07[b]	13

Slices were electrically stimulated (3 Hz, for 2 min) at the 45th and 75th min of superfusion. [a]Significantly different from the corresponding control, $P<0.05$, Mann-Whitney U-test; [b]Significantly different from the corresponding control, $P<0.025$, Mann-Whitney U-test.

whole experiment (Table 1). Thus, this compound behaved as a partial agonist (intrinsic activity $\alpha=0.35$). Its effect was not prevented by NX (NX 1 μM+[F/G]NC(1-13)NH_2 1 μM: St_2/St_1 0.73±0.10, $n=6$, $P<0.05$ vs NX alone, see Table 1).

Naloxone benzoylhydrazone (NBH) (3 μM) added to the medium at the 70th min of superfusion, together with NX (10 μM), did not modify [^{3}H]-5-HT efflux (St_2/St_1 0.92±0.12, $n=4$), but cancelled the effect of NC when both antagonists were present in the medium from the onset of superfusion (Table 1).

In some experiments slices were superfused with a medium containing a cocktail of peptidase inhibitors (captopril, phosphoramidon, bestatin, amastatin, all at 30 μM concentration, Nicholson *et al*., 1998) in order to test the relevance of enzymatic breakdown of NC. In these experimental conditions, the efflux of [^{3}H]-5-HT in St_1 evoked by 3 Hz stimulation was not different from slices superfused with normal medium, nor was the inhibitory effect of NC (0.1–0.3 μM) significantly potentiated (Table 2).

Discussion

The electrical stimulation-induced tritium efflux from rat cerebral cortex slices prelabelled with [^{3}H]-5-HT is frequency-related and Na^+- and Ca^{2+}-dependent and thus may be considered to represent a quasi-physiological 5-HT release (Goethert & Schlicker, 1991).

The present data clearly indicate that, in the rat cerebral cortex, ORL_1 functional sites inhibit 5-HT release. Although a high homology exists between ORL_1 and opioid receptors, NC does not bind to the latter (Meunier, 1997); in addition, the inhibitory action elicited by NC was not affected by the opioid receptor antagonist naloxone, ruling out the involvement of opioid receptors. Since in the cerebral cortex 5-HT is present exclusively in nerve terminals (Mamounas *et al*., 1992), the inhibitory effect of exogenous NC on 5-HT release, also observed in our laboratory in synaptosomal preparations (Siniscalchi *et al*., 1999), has to be ascribed to the activation of presynaptic heteroreceptors.

[F/G]NC(1-13)NH_2, which acts as an ORL_1 antagonist in the guinea-pig ileum, in the mouse and in the rat vasa deferentia (Guerrini *et al*., 1998; Bigoni *et al*., 1999), displayed an inhibitory effect on electrically evoked [^{3}H]-5-HT as well as [^{3}H]-noradrenaline efflux from cerebral cortex slices (present results, Schlicker *et al*., 1998; Werthwein *et al*., 1999), suggesting that this compound actually behaves as a partial agonist, possibly depending on different amounts of spare receptors in different tissues. Accordingly, in the experiments where [F/G]NC(1-13)NH_2 and NC (at maximally active concentrations) were added together to the medium before St_2, the observed inhibition was similar to that induced by [F/G]NC(1-13)NH_2 alone; on the other hand, when the latter drug was present in the medium throughout the whole experiment, it was able to prevent the effect of NC, added before St_2 (see also Schlicker *et al*., 1998).

In order to confirm the exclusive involvement of ORL_1 receptors in NC action, other experiments were carried out with the nonselective, competitive opioid/ORL_1 antagonist NBH in the presence of NX, at a concentration (10 μM) which was far in excess to block opioid receptors. Under these experimental conditions, NBH completely prevented NC-induced inhibition on [^{3}H]-5-HT efflux. Interestingly, NBH

did not modify 5-HT release *per se*, suggesting the lack of an inhibitory tone by endogenous NC on serotonergic terminals.

The low potency displayed by NC in the present work ($pEC_{50} = 6.54$) in comparison with data obtained by others (Nicol *et al*., 1996) was unexpected. This difference could be due to the enzymatic breakdown of the peptide, occurring in the absence of peptidase inhibitors (Nicholson *et al*., 1998). However, (a) our experiments were carried out in the presence of EDTA, which has been shown to efficiently prevent peptide breakdown (Montiel *et al*., 1997), and (b) NC did not display a significantly higher potency in the presence of a cocktail of peptidase inhibitors which has been shown to produce a leftward shift in concentration-response curves to NC in peripheral tissues (Nicholson *et al*., 1998; Okawa *et al*., 1999). Therefore, the reasons for the discrepancies seem to depend on the different model (central vs peripheral tissue) and/or experimental conditions (flow rate, thickness of the slices, type of stimulation). The bell-shaped concentration response curve obtained with NC, as well as with [F/G]NC(1-13)NH_2, in the present work, could be related to a rapid receptor desensitization, as shown by Morikawa *et al*. (1998) for Ca^{2+} channel inhibition in NG 108-15 cells: receptor desensitization in this way would be responsible for reduced efficacy at the highest concentrations. However, this hypothesis was challenged by the prolonged experiments including four stimulations (see Results), in which NC maintained its inhibitory effect (see also Werthwein *et al*., 1999). Once again, a high number of spare receptors in the cerebral cortex, substituting for the down-regulated ones, may be invoked to explain these conflicting results. A further explanation for the bell-shaped curve could be found in possible interactions by other neurotransmitters/modulators, occurring in complex experimental models (Jenck *et al*., 1997) and able to counteract the effect NC exerts on 5-HT release. Of course, this speculation requires proof.

In conclusion, an inhibitory action of NC on cortical 5-HT release has been clearly demonstrated in the present work, although its meaning remains to be clarified. Nevertheless, since the brain 5-hydroxytryptaminergic system has been implicated in states of anxiety (Bradley *et al*., 1992; Hamon, 1994) and NC has been reported to act as an effective agent in different behavioural models of anxiety (Jenck *et al*., 1997), it is tempting to suggest that this NC inhibition on 5-HT release may be a component of its anxiolytic action.

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