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Modulation of 5-hydroxytryptamine efflux from rat cortical synaptosomes by opioids and nociceptin

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1 The modulation of [^{3}H]-5-hydroxytryptamine ([^{3}H]-5-HT) efflux from superfused rat cortical synaptosomes by delta, kappa, mu and ORL_1 opioid receptor agonists and antagonists was studied.

2 Spontaneous [^{3}H]-5-HT efflux was reduced (20% inhibition) by either 0.5 μM tetrodotoxin or Ca^{2+}-omission. Ten mM K^+-evoked [^{3}H]-5-HT overflow was largely Ca^{2+}-dependent (90%) and tetrodotoxin-sensitive (50%).

3 The delta receptor agonist, deltorphin-I, failed to modulate the K^+-evoked neurotransmitter efflux up to 0.3 μM. The kappa and the mu receptor agonists, U-50,488 and endomorphin-1, inhibited K^+-evoked [^{3}H]-5-HT overflow (EC_{50} = 112 and 7 nM, respectively; E_{max} = 28 and 29% inhibition, respectively) in a norBinaltorphimine- (0.3 μM) and naloxone- (1 μM) sensitive manner, respectively. None of these agonists significantly affected spontaneous [^{3}H]-5-HT efflux.

4 The ORL_1 receptor agonist nociceptin inhibited both spontaneous (EC_{50} = 67 nM) and K^+-evoked (EC_{50} = 13 nM; E_{max} = 52% inhibition) [^{3}H]-5-HT efflux. The effect of NC was insensitive to naloxone (up to 10 μM), but was antagonized by [Nphe^1]nociceptin(1-13)NH_2 (a novel selective ORL_1 receptor antagonist; pA_2 = 6.7) and by naloxone benzoylhydrazone (pA_2 = 6.3). The ORL_1 ligand [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)NH_2 also inhibited K^+ stimulated [^{3}H]-5-HT overflow (EC_{50} = 64 nM; E_{max} = 31% inhibition), but its effect was partially antagonized by 10 μM naloxone.

5 It is concluded that the ORL_1 receptor is the most important presynaptic modulator of neocortical 5-HT release within the opioid receptor family. This suggests that the ORL_1/nociceptin system may have a powerful role in the control of cerebral 5-HT-mediated biological functions.
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Keywords: Synaptosomes; opioid receptors; nociceptin; 5-hydroxytryptamine; rat cerebral cortex; ORL_1; [Phe$^1\psi$(CH_2-NH)Gly^2]NC(1-13)NH_2: [Nphe^1]NC(1-13)NH_2

Abbreviations: DT1, deltorphin I; EM1, endomorphin 1; [F/G]NC(1-13)NH_2, [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)NH_2; NC, nociceptin; [Nphe^1]NC(1-13)NH_2, [Nphe^1]nociceptin(1-13)NH_2

Introduction

Cloning of the three 'classical' delta, kappa and mu opioid receptors (OP_1, OP_2 and OP_3, respectively, see Dhawan *et al*., 1996) led to the discovery of a novel member of this family: the 'opioid receptor like-1' (ORL_1) receptor (Mollereau *et al*., 1994). Activation of these receptors leads to neuronal hyperpolarization and inhibition of neurotransmitter release (for a review, see Meunier, 1997). Several endogenous peptides that act at these receptors have been identified, namely the enkephalins, endorphins, dynorphins (Bradbury *et al*., 1976; Hughes *et al*., 1975; Goldstein *et al*., 1979) and, more recently, endomorphins (Zadina *et al*., 1997) and nociceptin/orphanin FQ (NC; Meunier *et al*., 1995; Reinscheid *et al*., 1995). Enkephalins, endorphins and dynorphins do not display high selectivity for a single type of opioid receptor whereas endomorphins and NC bind with high affinity and selectivity to mu and ORL_1 receptors, respectively.

Opioid receptors are involved in a variety of central processes including nociception, locomotion, motivation, stress, anxiety, learning and feeding (for reviews, see Pasternak, 1993; Meunier, 1997). Accordingly, 'classical' opioid (for reviews, see Mansour *et al*., 1995; Simonato, 1996) and ORL_1 (Bunzow *et al*., 1994) receptors are widely distributed throughout the CNS and, in particular, all four receptors are found in the neocortex. Surprisingly, although they share similar post-receptor mechanisms, opposing/opposite effects mediated by these receptors have been observed. For example, the kappa receptor displayed anti-mu actions in various brain areas (for a review, see Pan, 1998). Likewise, intracerebroventricular injection of NC induced hyperalgesia while mu agonists induced analgesia (Meunier *et al*., 1995; Reinscheid *et al*., 1995). This functional antagonism may be ascribed to the inhibitory modulation of distinct neuronal types within a particular brain region, as suggested by the finding that opioid receptors are found on different nerve fibres and terminals (Mointeillet-Agius *et al*., 1998).

Superfused synaptosomal preparations represent a useful tool with which to investigate the distribution of a particular receptor on different neuronal types since it allows a direct modulation of neurotransmitter efflux to be studied. In the cerebral cortex, opioid modulation of neurotransmitter efflux from synaptosomes has focused on the release of [^{3}H]-noradrenaline (Mulder *et al*., 1987) or glutamate and GABA (Sbrenna *et al*., 1999a). In addition, we have reported data from a preliminary study of 5-HT efflux from cortical synaptosomes (Sbrenna *et al*., 1999b).

In order to address possible presynaptic modulation of serotonin release by selective 'classical' and ORL_1 opioid receptor agonists and antagonists, [^{3}H]-5-HT efflux from cortical synaptosomes was investigated. This will aid the

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completion of a detailed 'cortical map' of the presynaptic role of these receptors in the modulation of various neurotransmitters.

In the present study the effects of NC on [^{3}H]-5-HT efflux have been characterized using the ORL_1 ligand [Phe$^1\psi$(CH_2-NH)Gly^2]nociceptin(1-13)NH_2 ([F/G]NC(1-13)NH_2; Guerrini *et al*., 1998), the non-selective ORL_1 antagonist naloxone benzoylhydrazone (NalBzOH; Gistrack *et al*., 1989; Dunnill *et al*., 1996; Nicholson *et al*., 1998) and the novel pure (devoid of any residual agonist activity) and selective ORL_1 antagonist [Nphe^1]nociceptin(1-13) NH_2 ([Nphe^1]NC(1-13)NH_2; Guerrini *et al*., 1999; Calo *et al*., 2000).

Methods

Synaptosome preparation

Male Sprague-Dawley rats (180–240 g) were kept under standard conditions (12 h dark/light cycle, free access to food and water) and all procedures concerning animal treatment were in accordance with the current regulations governing animal experimentation in the U.K.

On the morning of the experiment, rats were decapitated under light ether anaesthesia and the fronto-parietal cortex was isolated. Synaptosomes were prepared as previously described (Morari *et al*., 1998). Briefly, the cortex was homogenized in ice-cold 0.32 M sucrose buffer at pH 7.4 then centrifuged for 10 min at $1000 \times g_{max}$ (4°C). The supernatant was then centrifuged for 20 min at $12{,}000 \times g_{max}$ (4°C) with the synaptosomal pellet being resuspended in oxygenated (95% O_2, 5% CO_2) Krebs solution (mM: NaCl 118.5, KCl 4.7, $CaCl_2$ 1.2, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 10) containing ascorbic acid (0.05 mM) and disodium EDTA (0.03 mM). Synaptosomes were pre-loaded with [^{3}H]-5-HT by incubation in medium containing 50 nM [^{3}H]-5-HT (specific activity 27.8 Ci mmol^{-1}, DuPont NEN) for 20 min.

One millilitre aliquots of the suspension (protein concentration of 0.35 mg protein ml^{-1}) were slowly injected into nylon syringe filters (outer diameter 13 mm, 0.45 μM pore size, internal volume of about 100 μl; MSI, Westporo, MA, U.S.A.) which were then connected to a peristaltic pump. Filters were maintained at 36.5°C in a thermostatic bath and superfused at a flow rate of 0.3 ml min^{-1} with a pre-oxygenated Krebs solution. Sample collection (every 3 min) was initiated after a 25 min period of filter washout. From the 25th to the 52nd min of sample collection (nine samples), spontaneous neurotransmitter efflux declined in a linear fashion (as shown in Figure 1A). Under these experimental conditions, spontaneous [^{3}H]-5-HT efflux was essentially unaffected by re-uptake. Indeed cumulative fractional [^{3}H]-5-HT release over a 40 min period (45%) was similar to that observed by Raiteri in his classical superfusion apparatus (Raiteri *et al*., 1974; Maura *et al*., 1982). K^+ stimulation (1 min pulse) was applied at the 43rd min. Agonists were added to the superfusion medium 12 min before the K^+ pulse and maintained until the end of the experiment. Antagonists were added 3 min before agonists.

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

At the end of the experiment, superfusate (3 min samples) and filter retained (dissolved with 1 ml of 1 M NaOH followed by 2 ml of 1 M HCl) radioactivity was determined by liquid scintillation spectrophotometry using a Beckman LS 1800 β-spectrometer and Ultima Gold XR scintillation fluid (Packard Instruments B.V., Groningen, The Netherlands). The chemical

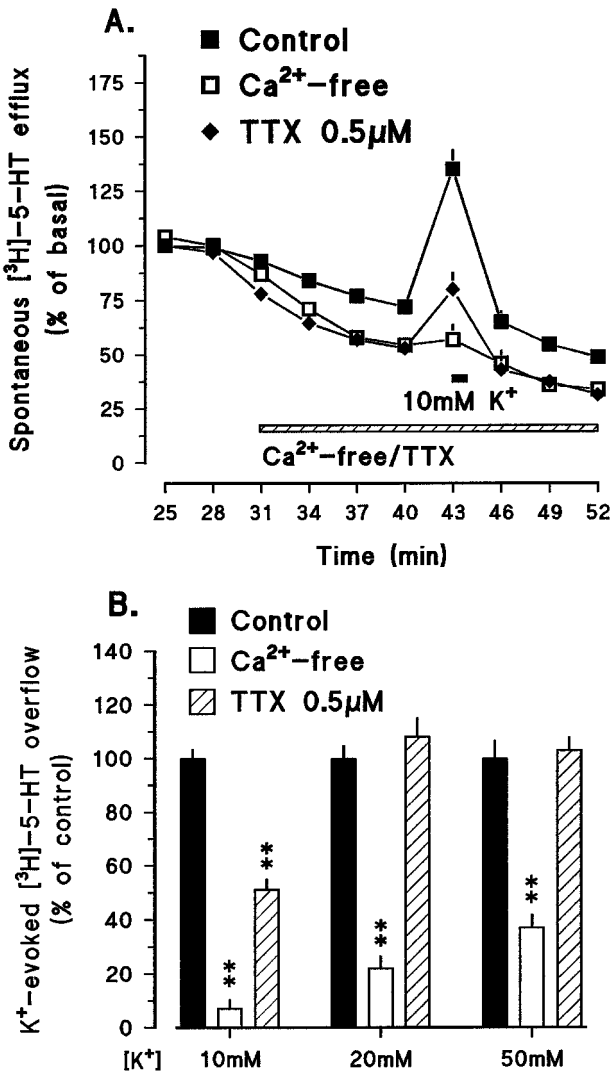

Figure 1 (A) Time course of [^{3}H]-5-HT efflux from cortical synaptosomes. Sample collection (every 3 min) was started after a 25 min period of filter wash-out. From the 25th to the 52nd min time-point samples (nine samples), spontaneous [^{3}H]-5-HT efflux showed a linear decline. K^+ stimulation (10 mM K^+; 1 min pulse; close bar) was applied at the 43rd min. Any tested treatment (as shown for Ca^{2+}-omission and tetrodotoxin) was started 12 min before the K^+ pulse and maintained until the end of experiment. (B) Effect of Ca^{2+}-free or 0.5 μM TTX containing medium on 10, 20 and 50 mM K^+-evoked [^{3}H]-5-HT overflow. Data are presented as a per cent of neurotransmitter overflow under control conditions (i.e. 10, 20 and 50 mM K^+ pulses). Data are means±s.e.mean of at least seven experiments. $^{**}P<0.01$ from control.

nature of the released radioactivity from superfused rat brain synaptosomes in the absence of monoamine oxidase inhibitors was initially investigated by Collard and colleagues (Collard *et al*., 1981), who demonstrated that during high K^+ stimulation, [^{3}H] overflow was almost exclusively 5-HT.

Drugs

All the peptides used in this study were prepared by solid-phase synthesis and purified by HPLC, as previously described (Calò *et al*., 1998a; Guerrini *et al*., 1999. Naloxone, U-50, 488 and norBinaltorphimine were purchased from Tocris Cookson (Bristol, U.K.) while NalBzOH from Research Biochemical Incorporated (Natick, MA, U.S.A.)

Stock solutions of each drug (1 mM) were made in distilled water and kept at −20°C until use.

Data presentation and statistical analysis

Data are expressed as: (i) percentages of basal neurotransmitter efflux, defined as the amount of neurotransmitter collected in the 28 min time-point sample (i.e. between the 28th and 31st min after the onset of superfusion; Figure 1A) or, when antagonists were used, in the 25 min time-point sample; (ii) fractional release, calculated as a percentage of the total radioactivity present in the filter at the beginning of the collection of fractions. Changes in spontaneous neurotransmitter efflux were evaluated by comparing the amount of neurotransmitter collected in the 40 min time-point sample (i.e. from the 12th to the 15th min after the onset of a particular treatment; Figure 1). K^+-evoked neurotransmitter overflow was calculated by subtracting the estimated spontaneous efflux (obtained by interpolation between the samples preceding and following the stimulation) from the total efflux observed in the stimulated sample (i.e. the 43 min time-point sample; Figure 1). When a treatment affected K^+-evoked neurotransmitter overflow, the effect was expressed as per cent of control stimulation.

Statistical analysis of absolute values was performed by analysis of variance (ANOVA) followed by the Newman-Keuls test for multiple comparisons. When only two groups of data were compared, the Student's *t*-test for unpaired data was used. Statistical analysis of per cent values was performed by the Kruskal-Wallis test (non-parametric ANOVA) followed by the Mann-Whitney *U*-test (including the Bonferroni's correction) for multiple comparisons.

The pharmacological terminology adopted in this paper is consistent with IUPHAR recommendations (Jenkinson *et al*., 1995): (i) EC_{50}, the molar concentration of an agonist that produces 50% of the maximal effect, calculated according to the standard logistic equation: $E = E_{min} + (E_{max} - E_{min})/(1 + (X/EC_{50})^n$; (ii) E_{max}, the maximal effect induced by an agonist; and (iii) pA_2, the negative logarithm to base 10 of the molar concentration of an antagonist that makes it necessary to double the agonist concentration to elicit the original submaximal response. pA_2 was calculated for NalBzOH and [Nphe^1]NC(1-13)NH_2 using the Gaddum Schild equation: $pA_2 = -\log((CR-1)/[\text{Antagonist}]$, assuming a slope equal to unity (CR is the ratio between the EC_{50} of NC calculated in the presence and in the absence of antagonist).

Results

Spontaneous neurotransmitter efflux

Basal [^{3}H]-5-HT efflux (6.4 ± 0.1 fmol mg protein^{-1} min^{-1}) corresponded to a fractional release of 3.5 ± 0.1%. Under control conditions, filter wash out caused a linear decline in spontaneous neurotransmitter efflux, reaching 73% of basal in the 40 min time-point sample, see also Figure 1A). Superfusion with both Ca^{2+}-free buffer or 0.5 μM tetrodotoxin (TTX) produced a 20% inhibition of spontaneous [^{3}H]-5-HT efflux (Figure 1A).

Modulation by opioid receptors Neither deltorphin-I (DT1; the selective delta receptor agonist), U-50,488 (the selective kappa receptor agonist), nor endomorphin-1 (EM1; the selective mu receptor agonist) significantly modified spontaneous [^{3}H]-5-HT efflux (Figure 2), while NC (0.001 – 1 μM), the selective ORL_1 receptor agonist, produced a concentration-dependent inhibition (Figure 2). The inhibitory effect of NC was significant at 0.1 μM and maximal at 1 μM, with an EC_{50} of 67 nM. Nal 10 μM, (ineffective alone, data not shown), did not significantly modify the effect of 1 μM NC (63.3 ± 1.3 and 63.5 ± 1.1% of basal, in the presence and in the absence of Nal, respectively; $n = 8$ for each group).

Stimulated neurotransmitter overflow

The effects of various K^+ concentrations on [^{3}H]-5-HT overflow (absolute values) are summarized in Table 1. [^{3}H]-5-HT overflow increased progressively within the 10 – 50 mM K^+ concentration range. The effect of higher (i.e. 75 mM) K^+ concentrations (68.2 ± 4.0 fmol mg protein^{-1} min^{-1}, $n = 6$), was not significantly different from that evoked by 50 mM K^+ (data not shown).

[^{3}H]-5-HT overflow evoked by 10 mM K^+ was Ca^{2+}-dependent (90%) and TTX-sensitive (50%, Figure 1B).

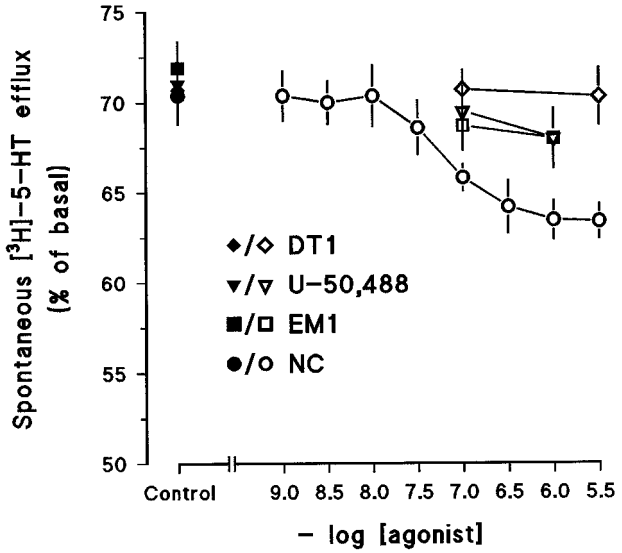

Figure 2 Concentration-response curves of the inhibitory effects of selective opioid receptor agonists on spontaneous [^{3}H]-5-HT efflux. Neurotransmitter efflux was inhibited in a concentration-dependent fashion by nociceptin (NC; statistically significant inhibition at 0.1 μM NC), while deltorphin I (DT1), U-50,488 and endomorphin 1 (EM1) were ineffective. Data are means ± s.e.mean of at least eight experiments.

Table 1 Effects of NC and TTX on [^{3}H]-5HT overflow evoked by different K^+ concentrations

K^+ (mM)	*Control* (fmol mg prot^{-1} min^{-1})	*NC* (1 μM) (fmol mg prot^{-1} min^{-1})	*TTX* (0.5 μM) (fmol mg prot^{-1} min^{-1})	*NC + TTX* (fmol mg prot^{-1} min^{-1})
10	9.3 ± 0.4	4.6 ± 0.2**	5.0 ± 0.3**	2.6 ± 0.1[a]
20	30.0 ± 1.4	25.5 ± 1.7*	32.8 ± 2.2	–
50	60.1 ± 2.4	64.2 ± 2.5	62.4 ± 3.6	–

[^{3}H]-5HT overflow is expressed as fmol mg protein^{-1} min^{-1}. Means ± s.e.mean of at least six experiments. *$P < 0.05$, **$P < 0.01$, from control. [a]$P < 0.01$ from 1 μM NC.

Conversely, [^{3}H]-5-HT overflow evoked by higher K^+ concentrations was completely insensitive to TTX and there was a reduction in the Ca^{2+}-dependent component (Figure 1B).

Modulation by opioid receptors

DT1 failed to significantly modify [^{3}H]-5-HT efflux evoked by 10 mM K^+ up to 1 μM (Figure 3), whilst there was a clear concentration-dependent inhibition with all the other agonists tested (Figure 3).

U-50,488 (1 nM – 3 μM) maximally inhibited [^{3}H]-5-HT overflow at 3 μM (−28% of control values) with an EC_{50} value of 112 nM. NorBNI (0.3 μM), the selective kappa receptor antagonist, which had no effect alone (data not shown), fully antagonized the effect of 1 μM U-50,488 (Table 2).

EM1 potently inhibited [^{3}H]-5-HT overflow (Figure 3) displaying an EC_{50} value of 7 nM and maximal inhibition of around 29%. The exogenous mu preferring agonist, morphine, tested at the single concentration of 1 μM, also inhibited [^{3}H]-5-HT overflow (Table 2). Ten μM Nal, which was ineffective alone (Figure 5B), fully antagonized the effect of both 0.1 μM EM1 and 1 μM morphine (Table 2).

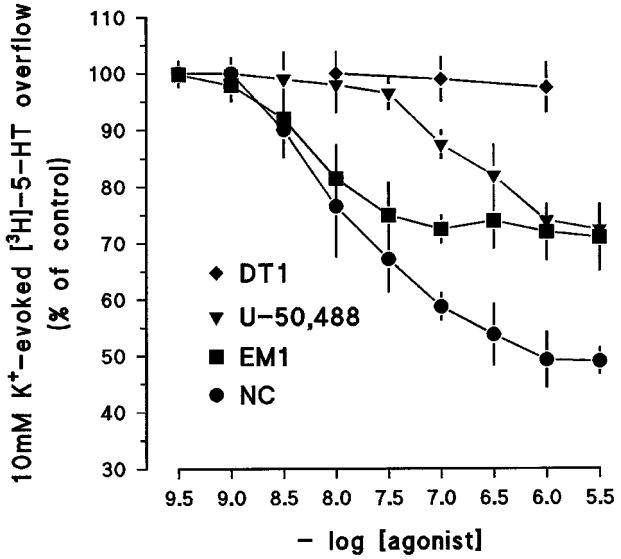

Figure 3 Concentration-response curves of the inhibitory effects of selective opioid receptor agonists on 10 mM K^+-evoked [^{3}H]-5-HT overflow. [^{3}H]-5-HT overflow was inhibited in a concentration-dependent fashion by U-50,488 (significant inhibition at 0.1 μM), endomorphin 1 (EM1; significant inhibition at 10 nM) and nociceptin (NC; significant inhibition at 3 nM), while deltorphin I (DT1) was ineffective up to 1 μM. Data are means ± s.e.mean of at least eight experiments.

The inhibition evoked by NC (1 μM) was clear when [^{3}H]-5-HT overflow was evoked by 10 and 20 mM K^+ but was not observed when stimulation was achieved with higher K^+ concentrations (Table 1). Interestingly, the inhibitory effect of 1 μM NC on 10 mM K^+-evoked overflow was not significantly modified in the presence of 0.5 μM TTX, since the peptide still halved the TTX-insensitive component of 10 mM K^+-evoked [^{3}H]-5-HT overflow (Table 1). Analysis of the concentration-response curve for NC (0.3 nM – 3 μM) yielded an EC_{50} value of 13 nM (Figure 3). The threshold concentration of NC was 3 nM (−12.3 ± 3.2%) and the maximal effect was obtained at 1 μM (52% inhibition). NalBzOH antagonized the inhibition induced by NC (Figure 4) while Nal (10 μM) was ineffective (Figure 5B). Since NalBzOH is also a kappa receptor agonist and a mu receptor mixed agonist/antagonist (Gistrak *et al*., 1989), 10 μM Nal was routinely added to the medium to avoid concurrent activation of 'classical' opioid receptors. Under this experimental condition, NalBzOH alone did not modify spontaneous or stimulated [^{3}H]-5-HT efflux (data not shown), but shifted to the right in a parallel manner the concentration-response curve to NC without altering the maximal effect elicited by the peptide ($pA_2 = 6.3$). In addition, the effects of the novel pure (devoid of any residual agonist activity) ORL_1 antagonist [Nphe^1]NC(1-13)NH_2 (10 μM) were examined (Figure 4). In the absence of Nal, 10 μM [Nphe^1]NC(1-13)NH_3 alone did not significantly modify either spontaneous or stimulated [^{3}H]-5-HT efflux (data not shown), but shifted to the right the concentration-response curve of NC in a parallel manner without altering the maximal effect ($pA_2 = 6.7$).

[Phe$^1\psi$(CH$_2$-NH)Gly^2]NC(1-13)NH$_2$

[^{3}H]-5-HT overflow evoked by 10 mM K^+ was also inhibited by the ORL_1 ligand [F/G]NC(1-13)NH_2 (Figure 5A) with an EC_{50} of 64 nM and a maximal effect obtained at 3 μM (31% inhibition). The effect of 3 μM [F/G]NC(1-13)NH_2 (Figure 5B) was reduced to about 50% by Nal (10 μM) but was unaffected by norBNI (0.3 μM, data not shown). To study any interaction between NC and [F/G]NC(1-13)NH_2, submaximal NC (0.1 μM) and supramaximal [F/G]NC(1-13)NH_2 (3 μM) concentrations were co-applied both in the presence and in the absence of 10 μM Nal (Figure 5B). When co-applied in the absence of Nal, the observed inhibition was not significantly different from that produced by NC alone. Conversely, when 10 μM naloxone was added to the medium, the final inhibition was smaller than that produced by NC.

NC-EM1 interactions

Near-threshold and supramaximal concentrations of NC and EM1 were co-applied to evaluate ORL_1-mu receptor interac-

Table 2 Effects of opioid receptor antagonists on kappa and mu receptor agonist mediated inhibition of 10 mM K^+-evoked [^{3}H]-5HT overflow

	Control (Per cent of K^+ effect)	*Nor-BNI* (0.3 μM) (Per cent of K^+ effect)	*Nal* (1μM) (Per cent of K^+ effect)
U-50,488 (1 μM)	73.6 ± 6.01**	99.3 ± 3.1	–
EM1 (0.1 μM)	72.5 ± 2.0**	–	102.3 ± 3.1
Mor (1 μM)	82.3 ± 2.9**	–	103.4 ± 4.0

Data are expressed as per cent of 10 mM $K^\pm$-evoked [^{3}H]-5HT overflow. Means ± s.e.mean of at least six experiments. **$P < 0.01$ from control.

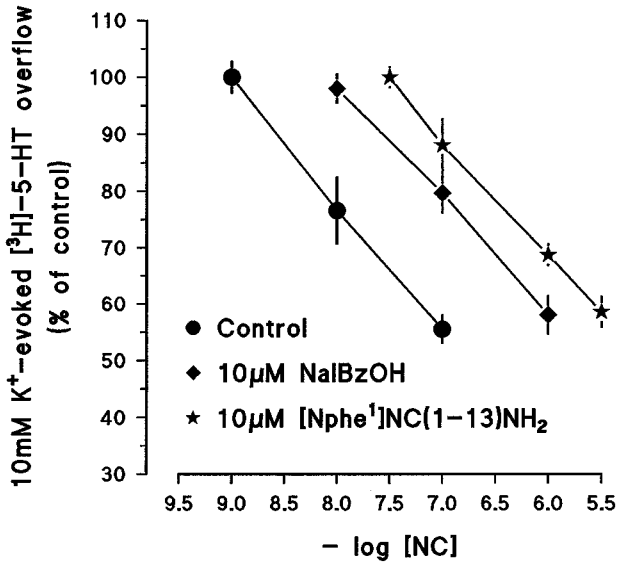

Figure 4 Effect of ORL_1 receptor antagonists on the nociceptin (NC) inhibition of 10 mM K^+-evoked [^{3}H]-5-HT overflow. The effect of 10 μM naloxone benzoylhydrazone (NalBzOH) (in the presence of 10 μM naloxone; Nal) and of 10 μM [Nphe^1]NC(1-13)NH_2 NC (in the absence of Nal) were shown. The concentration-response curve to NC was shifted to the right in a parallel manner without significant differences in the maximal effect. Data are means$\pm$s.e.mean of at least seven experiments.

tions in the modulation of [^{3}H]-5-HT overflow evoked by 10 mM K^+. Co-application of both agonists at 1 nM (ineffective alone) resulted in a significant inhibition of [^{3}H]-5-HT overflow (Figure 6A). In contrast, the inhibition obtained by co-application of both agonists at 1 μM did not exceed that observed with the more effective agonist alone (i.e. NC; Figure 6B).

Discussion

The present study demonstrated that K^+-evoked [^{3}H]-5-HT efflux from cortical synaptosomes was inhibited by kappa, mu and ORL_1 (but not delta) receptor agonists. Moreover, the ORL_1 receptor agonist NC also inhibited spontaneous [^{3}H]-5-HT efflux. In addition, use of the first selective ORL_1 receptor antagonist [Nphe^1]NC(1-13)NH_2 (devoid of any residual agonist activity) further substantiates the view that the effects of NC were mediated by ORL_1 receptor activation.

Spontaneous vs stimulated neurotransmitter efflux

Opioid agonists were tested against a 10 mM pulse of K^+, which was 90% Ca^{2+}-dependent and 50% TTX-sensitive, indicating the involvement of quasi-physiological release mechanisms. Conversely, the physiological relevance of the modulation of spontaneous neurotransmitter efflux is questionable. Indeed, only 20% of spontaneous [^{3}H]-5-HT efflux was both sustained by voltage-dependent Na^+ channel activation (TTX-sensitivity) and possibly by exocytotic release mechanisms (Ca^{2+}-dependency). Nevertheless, spontaneous firing of synaptosomes has been suggested in other studies (Marks *et al*., 1995; Tibbs *et al*., 1989; Morari *et al*., 1998; Sbrenna *et al*., 1999a). However, the fraction of spontaneous [^{3}H]-5-HT efflux that could be modulated was small and only NC inhibition of [^{3}H]-5-HT efflux reached significance.

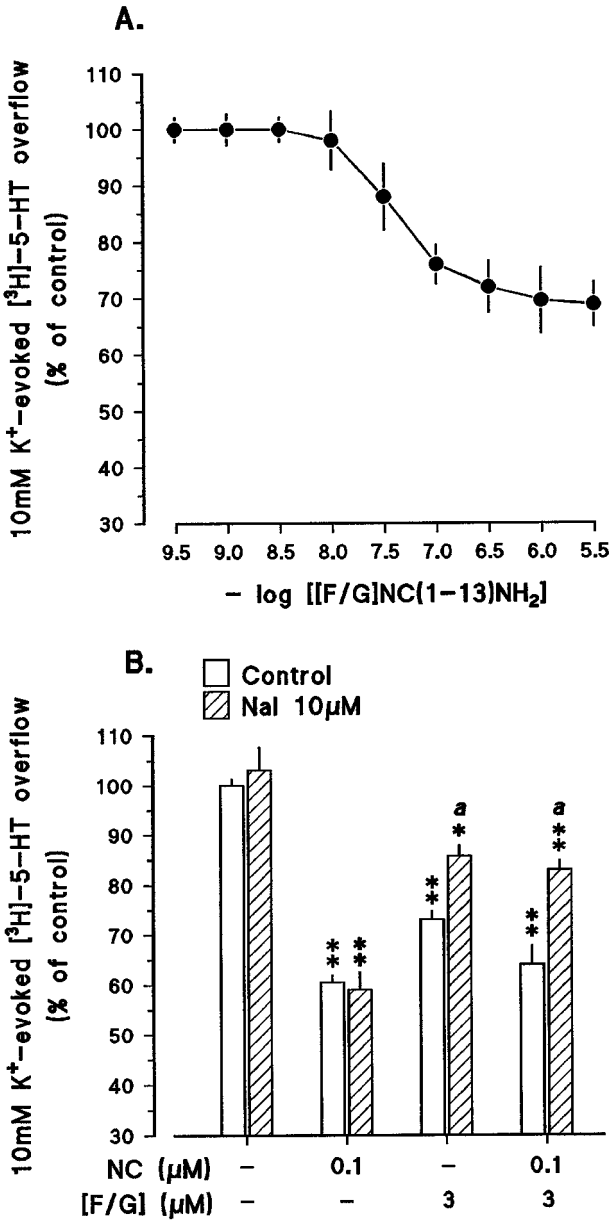

Figure 5 (A) Concentration-response curve for the inhibitory effect of [F/G]NC(1-13)NH_2 on 10 mM K^+-evoked [^{3}H]-5-HT overflow. Data are means$\pm$s.e.mean of at least seven experiments. (B) Effect of 0.1 μM NC and 3 μM [F/G]NC(1-13)NH_2 ([F/G]) alone or co-applied on 10 mM K^+-evoked [^{3}H]-5-HT overflow both in the presence and in absence of 10 μM naloxone (Nal). Nal did not modify the effects of NC, but partially antagonized the effects of [F/G]NC(1-13)NH_2. Co-application of 0.1 μM NC and 3 μM [F/G]NC(1-13)NH_2 in the presence of naloxone attenuated the inhibition produced by NC alone. Data are means$\pm$s.e.mean of at least six experiments. $^*P<0.05$ and $^{**}P<0.01$ from control and $^aP<0.05$ from the same treatment in the absence of Nal.

'Classical' opioid receptors

DT1 (Erspamer *et al*., 1989) was ineffective in modulating [^{3}H]-5-HT efflux, suggesting that serotonergic terminals in the adult rat cerebral cortex do not express a significant number of delta receptors. Since the delta receptor is expressed in rat fronto-parietal cortex (Mansour *et al*., 1995), terminals other than serotonergic (possibly dopaminergic; Tooyama *et al*., 1993) may be modulated by DT1.

Both U-50,488 (Von Voigtlander *et al*., 1983) and EM1 inhibited stimulated [^{3}H]-5-HT overflow. The exclusive

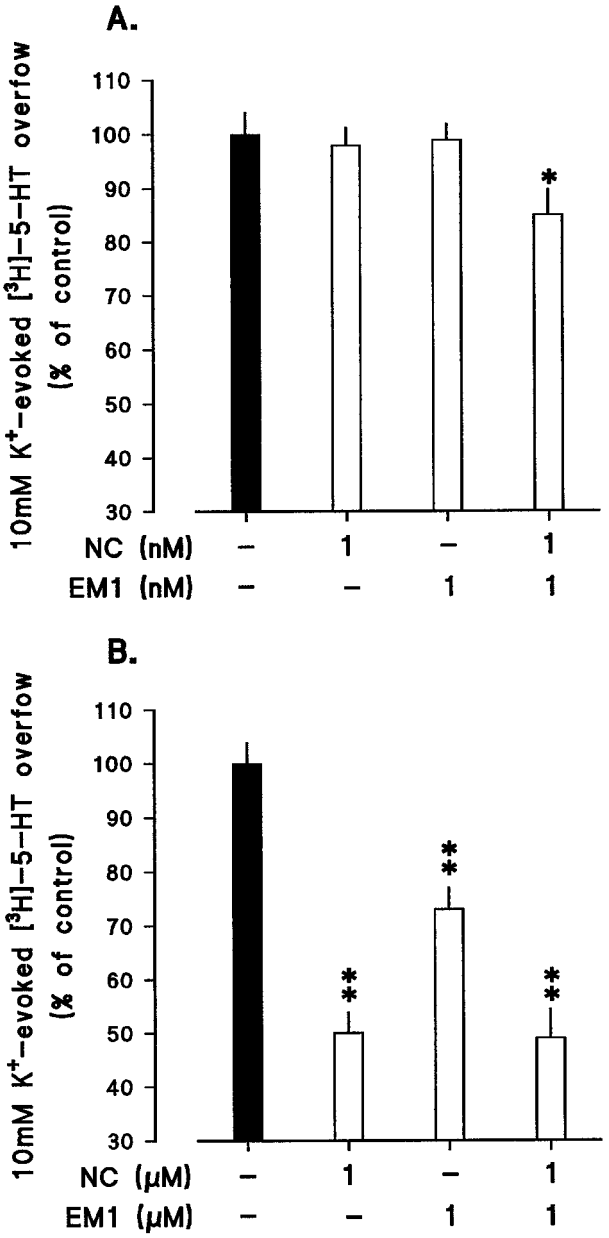

Figure 6 (A) Effect of co-application of subthreshold concentrations of endomorphine 1 (EM1, 1 nM) and nociceptin (NC, 1 nM) on 10 mM K^+ -evoked [^{3}H]-5-HT overflow. The peptides were ineffective when applied alone but significantly inhibited [^{3}H]-5-HT overflow when co-applied. (B) Effect of co-application of supramaximal concentrations of EM1 (1 μM) and NC (1 μM) on 10 mM K^+-evoked [^{3}H]-5-HT overflow. When co-applied, the inhibition observed was not significantly greater than that obtained with the more effective peptide alone. Data are means ± s.e.mean of at least six experiments. $^{**}P<0.01$ from control.

involvement of kappa receptors in the effect of U-50,488 was confirmed by its complete sensitivity to norBNI. Moreover, the EC_{50} value for U-50,488 inhibition of $^{45}Ca^{2+}$ entry into rat cortical synaptosomes (80 nM; Xiang *et al*., 1990) was similar to that calculated in this study. The naloxone-sensitivity and the effective range of agonist concentration used suggested the exclusive involvement of mu receptors in the actions of EM-1 (Zadina *et al*., 1997). In support of this view, the exogenous mu-preferring agonist morphine, also inhibited [^{3}H]-5-HT overflow.

The present data represent the first evidence of a direct presynaptic modulation of serotonergic terminals by kappa and mu receptors in the neocortex and clearly indicate that both receptors are located on serotonergic nerve terminals in this brain area. At variance with the present data, Hagan & Hughes (1984) failed to demonstrate a modulation of [^{3}H]-5-HT efflux from cortical slices by morphine in the only *in vitro* study addressing this topic. Nevertheless, serotonergic transmission in the neocortex is known to play an important role in many biological functions, like nociception/analgesia (Driessen & Reimann, 1992; Meagher *et al*., 1989; Pini *et al*., 1997), stress/anxiety (Kawahara *et al*., 1993; Lewis *et al*., 1980; Petty *et al*., 1992), and food intake/anorexia (Kaye, 1997; Mercer & Holder, 1997) in which the opioid system also has been implicated. Therefore, the direct modulation of 5-HT efflux by opioid receptors may represent an important mechanism of interaction between serotonergic and opioidergic systems of relevance in the physiological and pathophysiological control of cerebral 5-HT-mediated functions.

ORL_1 receptor

The effect of NC was not seen when [^{3}H]-5-HT overflow was evoked by abrupt depolarization with K^+ 50 mM. Although this failure to observe an inhibition may be due to an inability to modulate an unphysiological depolarization, very high extracellular K^+ concentration could have also interfered with post-receptor mechanisms (involving, among others, the activation of an outward K^+ current; Meunier, 1997) triggered by the peptide. Interestingly, TTX did not modify the inhibition of 10 mM K^+-evoked [^{3}H]-5-HT overflow induced by NC, suggesting that the effects of NC and TTX are mediated by distinct mechanisms (i.e. K^+ channels activation/ Ca^{2+} channels inhibition and Na^+ channels blockade, respectively).

The effective concentrations of NC ($EC_{50}=13$ nM) and the insensitivity to Nal, clearly indicated that NC effect was not due to the concurrent activation of 'classical' opioid receptors (see Meunier *et al*., 1995; Reinscheid *et al*., 1995). Furthermore, by using [Nphe^1]NC(1-13)NH_2 we demonstrated that the effect of NC was mediated *via* the ORL_1 receptor. This new compound has been reported to bind selectively to recombinant ORL_1 receptors and to antagonize NC-induced inhibition of forskolin stimulated cyclic AMP accumulation in CHO_{ORL1} cells ($pA_2=6.0$, Calo *et al*., 2000; $pA_2=6.2$, Hashimoto *et al*., 2000). It is worthy of mention that [Nphe^1]NC(1-13)NH_2 acted as a ORL_1 selective antagonist devoid of any residual agonist activity on native NC receptors in a variety of NC-sensitive peripheral tissues (pA_2 between 6.0 and 6.4, Calo *et al*., 2000; Rizzi *et al*., 1999) and *in vivo*, where it prevented the pronociceptive and antimorphine effects of NC in the mouse tail withdrawal assay (Calo *et al*., 2000) and the hyperphagic action of NC in the rat (Polidori *et al*., 2000). The present data extend these observations in that [Nphe^1]NC(1-13)NH_2 is also active at native central ORL_1 receptors, by antagonizing NC inhibition of [^{3}H]-5-HT release with a pA_2 value similar to those obtained previously (Calo *et al*., 2000; Hashimoto *et al*., 2000, Rizzi *et al*., 1999). In support of this view was the finding that NalBzOH, a non-selective ORL_1 receptor antagonist (Dunnill *et al*., 1996; Nicholson *et al*., 1998), competitively antagonized the effect of NC on K^+-evoked neurotransmitter efflux, with a pA_2 value (6.3) in line with that calculated in other studies of different tissue preparations (rat vas deferens, $pA_2=6.3$ and 6.6 according to Nicholson *et al*. (1998) and Dunnill *et al*. (1996), respectively; mouse brain slices, $pA_2=6.6$ according to Schlicker *et al*. (1998).

Previous studies in brain slice preparations reported that glutamate (Nicol *et al*., 1996) and noradrenaline (Schlicker *et*

al., 1998) release was inhibited by NC with identical EC_{50} values (51 and 55 nM, respectively) in the presence or in the absence of peptidase inhibitors. Peptidase inhibitors were not used in the present study since: (i) they did not appear to be essential for the effect of NC; (ii) the superfused synaptosome monostratum allows the peptide to directly reach ORL_1 receptors; and (iii) the use of 30 μM EDTA in the perfusion medium is expected to prevent, at least in part, NC degradation (Montiel *et al*., 1997). The present data suggest that the inhibitory effect exerted by NC on [^{3}H]-5-HT overflow from cerebral cortex slices is largely dependent upon activation of ORL_1 presynaptic receptors (Siniscalchi *et al*., 1999; Werthwein *et al*., 1999).

[F/G]NC(1-13)NH$_2$

This synthetic peptide, initially described as a selective ORL_1 receptor antagonist (Guerrini *et al*., 1998), was subsequently found to display partial to full agonist activity at the ORL_1 receptor in various tissue preparations and *in vivo* (Schlicker *et al*., 1998; Carpenter & Dickenson, 1998; Calo *et al*., 1998b; Okawa *et al*., 1999), possibly reflecting differing ORL_1 receptor density in various preparations (Toll *et al*., 1998). In the present study, [F/G]NC(1-13)NH_2 inhibited [^{3}H]-5-HT stimulated overflow with a lower E_{max} than NC, thus acting as a partial agonist at ORL_1 receptors. Accordingly, in the presence of Nal, coapplication of 0.1 μM NC and 3 μM [F/G]NC(1-13)NH_2 resulted in a decrease in the overall inhibition of [^{3}H]-5-HT overflow, suggesting that [F/G]NC(1-13)NH_2 bound to ORL_1 receptors reducing the efficacy of the full agonist NC. Indeed, in the absence of Nal, this was not evident possibly due to concurrent activation of 'classical' opioid receptors (probably mu receptors) by [F/G]NC(1-13)NH_2. This was also shown in our previous report on synaptosomal glutamate and GABA release (Sbrenna *et al*., 1999a).

To date [F/G]NC(1-13)NH_2 has not been reported to interact with 'classical' opioid receptors (up to 10 μM) in various *in vitro* bioassays (Calo *et al*., 1998a). Nevertheless, a recent binding study (Varani *et al*., 1999) demonstrated only a 60 fold ORL_1/mu selectivity for [F/G]NC(1-13)NH_2 in guinea-pig brain membranes, with a K_i for the mu receptor of some 800 nM. Moreover, an electrophysiological study in the rat spinal cord (Carpenter & Dickenson, 1998) also reported that the effects of [F/G]NC(1-13)NH_2 are partially sensitive to Nal, suggesting that, at least in CNS preparations, the synthetic peptide may not be selective for ORL1 receptors.

NC-EM1 interactions

When co-applied, subthreshold concentrations of NC and EM1, both ineffective alone, produced a synergistic inhibition of K^+-stimulated [^{3}H]-5-HT overflow. However, inhibition of [^{3}H]-5-HT overflow obtained with supramaximal concentrations of the two agonists was not significantly different from that evoked by the more effective agonist alone. These data are in line with the finding that 'classical' opioid and ORL_1 receptors share similar post-receptor mechanisms (Meunier, 1997), and suggest that both a synergistic activation and a ceiling effect of these post-receptor transduction mechanisms can be observed. As both ORL_1 and 'classical' opioid receptor agonists displayed identical qualitative effects (i.e. inhibitory) on neurotransmitter release, the only conceivable mechanism leading to functional opposite effects is a peculiar arrangement of terminals lacking ('non-responsive') or expressing ('responsive') these receptors within a particular brain area. This view was corroborated by comparing the present data with our previous report showing the ineffectiveness of NC in modulating glutamatergic and GABAergic terminals in the rat neocortex (Sbrenna *et al*., 1999a). It is therefore not surprising that this peptide can produce both antinociceptive (opioid-like) and pronociceptive effects in the rat at spinal and supraspinal sites, respectively, possibly reflecting a modulation of different neuronal types (for a review see Meunier, 1997).

In conclusion, the ORL_1 receptor appears to be the most important presynaptic modulator of neocortical serotonin release within the opioid receptor family. Moreover, by comparing the present data with our previous report (Sbrenna *et al*., 1999a), the existence of important differences in term of cell type sensitivity to NC in the neocortex emerges.

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