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RESEARCH PAPER

Nocistatin inhibits 5-hydroxytryptamine release in the mouse neocortex via presynaptic $G_{i/o}$ protein linked pathways

M Fantin[1], C Fischetti[1], C Trapella[2] and M Morari[1]

[1]*Department of Experimental and Clinical Medicine, Section of Pharmacology, Istituto Nazionale di Neuroscienze, University of Ferrara, Ferrara, Italy and* [2]*Department of Pharmaceutical Sciences and Biotechnology Center, University of Ferrara, Ferrara, Italy*

Background and purpose: Nocistatin (NST) is a neuropeptide generated from cleavage of the nociceptin/orphanin FQ (N/OFQ) precursor. Evidence has been presented that NST acts as a functional antagonist of N/OFQ, although NST receptor and transduction pathways have not yet been identified. We previously showed that N/OFQ inhibited [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) release from mouse cortical synaptosomes via activation of NOP receptors. We now investigate whether NST regulates [^{3}H]5-HT release in the same preparation.
Experimental approach: Mouse and rat cerebrocortical synaptosomes in superfusion, preloaded with [^{3}H]5-HT and stimulated with 1 min pulses of 10 mM KCl, were used.
Key results: Bovine NST (b-NST) inhibited the K^+-induced [^{3}H]5-HT release, displaying similar efficacy but lower potency than N/OFQ. b-NST action underwent concentration-dependent and time-dependent desensitization, and was not prevented either by the NOP receptor antagonist [Nphe^1 Arg^{14},Lys^{15}]N/OFQ(1-13)-NH_2 (UFP-101) or by the non-selective opioid receptor antagonist, naloxone. Contrary to N/OFQ, b-NST reduced [^{3}H]5-HT release from synaptosomes obtained from NOP receptor knockout mice. However, both N/OFQ and NST were ineffective in synaptosomes pre-treated with the $G_{i/o}$ protein inhibitor, *Pertussis* toxin. NST-N/OFQ interactions were also investigated. Co-application of maximal concentrations of both peptides did not result in additive effects, whereas pre-application of maximal b-NST concentrations partially attenuated N/OFQ inhibition.
Conclusions and implications: We conclude that b-NST inhibits [^{3}H]5-HT release via activation of $G_{i/o}$ protein linked pathways, not involving classical opioid receptors and the NOP receptor. The present data strengthen the view that b-NST is, *per se,* a biologically active peptide endowed with agonist activity.
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Keywords: 5-HT release; nociceptin/orphanin FQ; nocistatin; NOP receptor knockouts; pertussis toxin; UFP-101; synaptosomes

Abbreviations: b-NST, bovine nocistatin; FR, fractional release; J-113397, 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H* benzimidazol-2-one; N/OFQ, nociceptin/orphanin FQ; NOP, N/OFQ opioid peptide; $NOP^{-/-}$, NOP receptor knockout; NST, nocistatin; PTX, *Pertussis* toxin; TTX, tetrodotoxin; UFP-101, [Nphe^1, Arg^{14}, Lys^{15}]N/OFQ(1–13)-NH_2

Introduction

Nocistatin (NST) is a biologically active neuropeptide generated from the cleavage of the nociceptin/orphanin FQ (N/OFQ) precursor (Okuda-Ashitaka *et al.*, 1998). In contrast to N/OFQ, only the C-terminal portion of the NST sequence is conserved among species, while the full peptide isolated from the bovine, human, rat and mouse brain contains 17, 30, 35 and 41 amino acids, respectively. Bovine NST (b-NST; b-PNP-3) was originally described as a functional antagonist of N/OFQ, devoid of primary agonist activity. In mice, b-NST blocked the induction of hyperalgesia and allodynia induced by intrathecal administration of N/OFQ or prostaglandin E_2 (Okuda-Ashitaka *et al.*, 1998) and reversed the N/OFQ inhibition of learning and memory (Hiramatsu and Inoue, 1999), whereas, in rats, it reversed the N/OFQ inhibition of K^+-stimulated cortical glutamate release (Nicol *et al.*, 1998) and attenuated the N/OFQ-induced hyperphagia (Olszewski *et al.*, 2000). A number of reports, however, have also shown

Correspondence: Professor M Morari, Department of Experimental and Clinical Medicine, Section of Pharmacology, and Neuroscience Center, University of Ferrara, via Fossato di Mortara 17-19, Ferrara 44100, Italy.
E-mail: m.morari@unife.it
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that b-NST can exert *per se* biological effects, such as reversal of scopolamine-induced impairment of learning and memory (Hiramatsu and Inoue, 1999), reduction of pain responses in phase I of the formalin test (Yamamoto and Sakashita, 1999), disinhibition of discharge rate of thalamic neurons (Albrecht *et al*., 2001), increased anxiety (Gavioli *et al*., 2002) and potentiation of electropuncture-induced analgesia (Huang *et al*., 2003). Although these effects may be due to interference with endogenous N/OFQ transmission, the finding that b-NST and N/OFQ inhibited different populations of synapses in the spinal cord suggested that b-NST can act also via N/OFQ-independent pathways (Zeilhofer *et al*., 2000; Ahmadi *et al*., 2001). Indeed, *in vitro* studies showed that b-NST bound to mouse brain and spinal cord membranes with high affinity, but it did not displace [^{3}H]N/OFQ binding nor did it prevent N/OFQ inhibition of forskolin-induced cyclic AMP accumulation in CHO cells transfected with the N/OFQ opioid peptide (NOP) receptor (Okuda-Ashitaka *et al*., 1998). The possibility that NST acts through a yet unidentified G protein-coupled receptor is supported by the finding that rat NST-induced suppression of inhibitory neurotransmission in the rat spinal cord *in vitro* (Zeilhofer *et al*., 2000) or b-NST-induced nociception *in vivo* (Inoue *et al*., 2003) is sensitive to the $G_{i/o}$ protein inhibitor, *Pertussis* toxin (PTX).

We previously showed that N/OFQ inhibited [^{3}H]5-hydroxytryptamine ([^{3}H]5-HT) release from rodent cortical synaptosomes via activation of NOP receptors (Sbrenna *et al*., 2000; Marti *et al*., 2003; Mela *et al*., 2004). We therefore sought to investigate whether and through which mechanisms b-NST regulates [^{3}H]5-HT release in a synaptosomal preparation from the mouse neocortex. In particular, we employed a genetic model (NOP receptor knockout mice; $NOP^{-/-}$ mice) and a pharmacological approach (receptor antagonists) to investigate the involvement of classical opioid and NOP receptors in the b-NST action. Moreover, we tested the PTX sensitivity of the b-NST effects to evaluate the involvement of $G_{i/o}$ protein-dependent transduction pathways. Finally, we investigated whether b-NST could modulate N/OFQ responses. The present study demonstrates that b-NST presynaptically inhibits [^{3}H]5-HT release via activation of $G_{i/o}$ protein-mediated mechanisms not involving classical opioid receptors and the NOP receptor.

Materials and methods

Animals

Male Swiss mice (20–25 g; Stefano Morini, Modena, Italy), and CD1/C57BL6J/129 $NOP^{+/+}$ and $NOP^{-/-}$mice were employed in these studies. $NOP^{+/+}$ and $NOP^{-/-}$ mice were generated on a mixed C57BL/6J and 129 genetic background (Nishi *et al*., 1997) and backcrossed with CD1 mice for nine generations (Marti *et al*., 2004). All animals were genotyped by PCR. Mice were kept under standard conditions (12 h dark:12 h light cycle, free access to food and water), and all procedures concerning animal treatment were in accordance with European Communities Council Directives (86/609/EEC) and National regulations (DL 116/92).

Preparation of synaptosomes

On the morning of the experiment, mice were decapitated under light ether anaesthesia and the fronto-parietal cortex was isolated. Synaptosomes were prepared as previously described (Sbrenna *et al*., 2000). Briefly, the cortex was homogenized in ice-cold 0.32 M sucrose buffer at pH 7.4 and centrifuged for 10 min at 1000 *g* (4°C). The supernatant was centrifuged for 20 min at 12 000 *g* (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 and glucose 10) containing ascorbic acid (0.05 mM) and disodium ethylenediamine tetraacetic acid (EDTA; 0.03 mM).

Synaptosomes were preloaded with [^{3}H]5-HT by incubation (25 min) in medium containing 50 nM [^{3}H]5-HT (specific activity of 30 Ci mmol^{-1}; NEN DuPont, Boston, MA, USA), and 0.8 ml aliquots of the suspension (protein concentration of approximately 0.35 mg protein ml^{-1}) were slowly injected into nylon syringe filters (inner diameter 13 mm, 0.45 μm pore size; internal volume approximately 100 μl; MSI, Westporo, MA, USA) connected to a peristaltic pump. Filters were maintained at 36.5°C in a thermostatic bath and superfused orthogonally at a flow rate of 0.4 ml min^{-1} with a preoxygenated Krebs solution. Sample collection (every 3 min) started after a 20 min period of filter washout. K^+ stimulation (1 min pulse) was applied at the 38th minute. Under these experimental conditions, the 10 mM K^+-evoked [^{3}H]5-HT overflow has been previously reported to be largely Ca^{2+}-dependent (~90%) and tetrodotoxin (TTX)-sensitive (~50%; Sbrenna *et al*., 2000; Mela *et al*., 2004). b-NST and N/OFQ were added to the superfusion medium 9 min before the K^+ pulse (unless otherwise stated). Antagonists were added 3 min before agonists and maintained until the end of the experiment.

When PTX was used, the toxin was entrapped into synaptosomes, that is, it was added to the sucrose buffer used for homogenization at a concentration of 5 nM (Raiteri *et al*., 2000). Following homogenization, synaptosomes were preincubated in Krebs solution containing PTX (5 nM) for 30 min at room temperature, and finally incubated with [^{3}H]5-HT (in the presence of PTX).

[^{3}H]5-HT analysis

At the end of the experiment, superfusate (3 min collection samples) and filter retained radioactivity (dissolved with 0.8 ml of 1 M NaOH followed by 0.8 ml of 1 M HCl and another 0.8 ml of 1 M NaOH) was determined by liquid scintillation spectrophotometry using a Beckman LS 1800 β-spectometer and Ultima Gold XR scintillation fluid (Packard Instruments BV, Groningen, The Netherlands).

Data presentation and statistical analysis

All data are expressed as means ± s.e. mean of *n* determinations. Data were calculated as fractional release (FR, that is, tritium efflux expressed as percentage of the tritium content in the filter at the onset of the corresponding collection period) and expressed as percentage of K^+-evoked tritium overflow. K^+-evoked tritium 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 and expressed as percentage of the tritium content in the filter at the onset of the corresponding collection period (NET FR). Statistical analysis was performed on NET FR values by analysis of variance, followed by the Newman–Keuls test for multiple comparisons.

Drugs

N/OFQ, [Nphe^1, Arg^{14}, Lys^{15}]N/OFQ(1–13)-NH$_2$ (UFP-101) and b-NST were prepared in the Department of Pharmaceutical Sciences at the Ferrara University (Calo *et al.*, 1998; Guerrini *et al.*, 2000). A batch of b-NST was also purchased from NeoMPS (Strasbourg, France) for a comparison. No difference in activity was observed between b-NST synthesized in Ferrara and that commercially available. PTX was purchased from Calbiochem (San Diego, CA, USA), naloxone from Tocris Cookson (Bristol, UK) and [^{3}H]5-HT from Perkin Elmer Life Sciences Inc. (Boston, MA, USA).

All drugs were dissolved in distilled water before use.

Results

Spontaneous [^{3}H]5-HT efflux (FR) from mouse cortical synaptosomes was $5.34 \pm 0.06\%$ ($n = 190$) and was not modified by pretreatment with N/OFQ and b-NST (data not shown). Conversely, tritium overflow (NET FR) evoked by 1 min pulse of 10 mM K^+ ($2.1 \pm 0.1\%$; $n = 46$) was inhibited in a concentration-dependent manner by both N/OFQ (0.001–10 μM; Figure 1a) and b-NST (0.001–30 μM; Figure 1a). Maximal inhibition was observed at 0.1 μM N/OFQ ($\sim$30% inhibition) and 1 μM b-NST ($\sim$26% inhibition). For both peptides, higher concentrations were found to be ineffective. To investigate whether the effects of b-NST were species-dependent, b-NST was tested in rat cortical synaptosomes (Figure 1b). b-NST inhibited the K^+-evoked tritium overflow showing maximal effect at 0.1 μM ($\sim$23%) and loss of efficacy at 10 μM. For a comparison, N/OFQ was also tested (Figure 1b). N/OFQ inhibited tritium overflow in the 1 nM–0.1 μM range, showing maximal efficacy at 0.1 μM ($\sim$40%) and progressive loss of efficacy at higher concentrations (Marti *et al.*, 2003; Mela *et al.*, 2004). As we had previously showed that the effects of N/OFQ were time-dependent (Mela *et al.*, 2004), the effect of a maximal b-NST concentration (1 μM) was studied under different pre-application times (6–21 min before K^+; Figure 2). b-NST caused essentially the

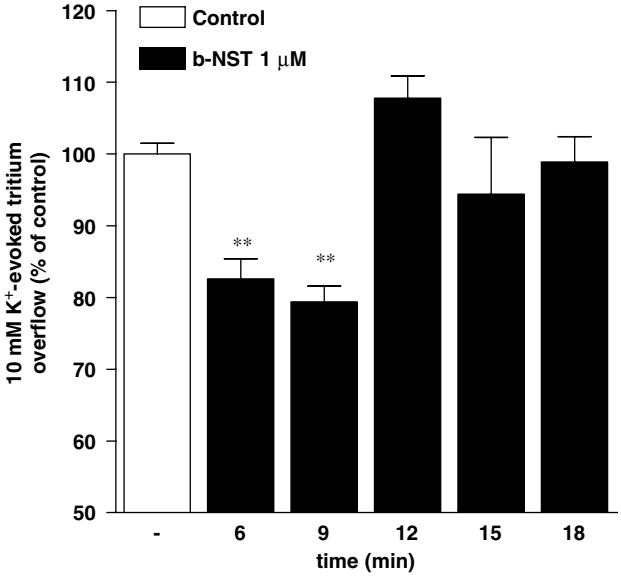

Figure 2 Time dependency of the bovine nocistatin (b-NST) effect. Effect of b-NST (1 μM) on the K^+-evoked [^{3}H]5-HT overflow in neocortical mouse synaptosomes. b-NST was applied for 6, 9, 12, 15 and 18 min before K^+ and maintained until the end of the experiment. Data are expressed as percentage of the K^+ stimulation (control) and are means $\pm$ s.e.m. of eight experiments. *$P < 0.05$, **$P < 0.01$; different from control (analysis of variance followed by the Newman–Keuls test for multiple comparisons).

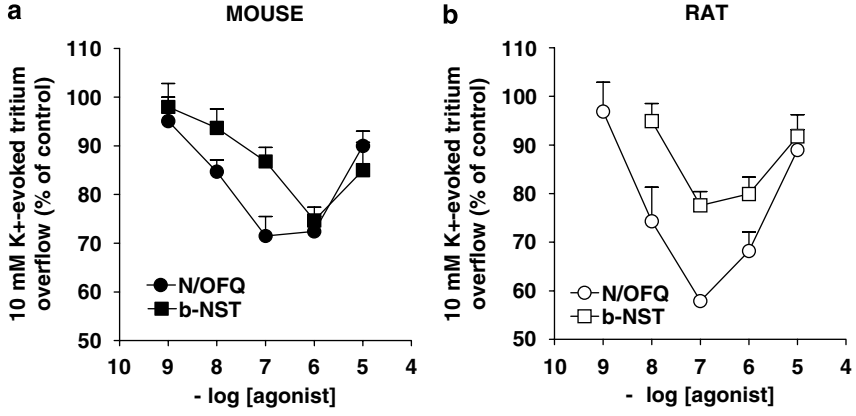

Figure 1 Nociceptin/orphanin FQ (N/OFQ) and bovine nocistatin (b-NST) inhibit [^{3}H]5-HT overflow from cortical synaptosomes. Concentration–response curves describing the effects of N/OFQ (0.001–10 μM) and b-NST (0.001–10 μM) on the K^+-evoked [^{3}H]5-HT overflow from mouse (**a**) and rat (**b**) neocortical synaptosomes. Maximally effective b-NST concentrations were 1 μM (mouse) and 0.1 μM (rat; analysis of variance followed by the Newman–Keuls test for multiple comparisons). Drugs were perfused 9 min before K^+ and maintained until the end of the experiment. Data are expressed as percentage of the K^+ stimulation (control) and are means $\pm$ s.e.m. of 10–20 determinations.

same extent of inhibition when pre-applied for 6 or 9 min. However, longer pre-application periods resulted in a loss of b-NST efficacy.

A pharmacological and genetic approach was then taken to study whether the effects of NST were due to activation of classical opioid or NOP receptors. First, b-NST was tested in the presence of a mixture of 1 μM naloxone (a non-selective opioid receptor antagonist) and 1 μM UFP-101 (a selective NOP receptor antagonist). Naloxone and UFP-101, ineffective alone, did not antagonize b-NST inhibition of the K^+-evoked tritium overflow (Figure 3a). Second, a maximally effective b-NST concentration (1 μM) was tested in synaptosomes obtained from $NOP^{-/-}$ mice (Figure 3b). Spontaneous and K^+-evoked [^{3}H]5-HT overflow from $NOP^{-/-}$ and $NOP^{+/+}$ mice was not different from that measured in Swiss mice. In $NOP^{+/+}$ and $NOP^{-/-}$ mice, spontaneous efflux (FR) was 5.8 ± 0.2 and 6.2 ± 0.1% respectively, while the K^+-evoked [^{3}H]-5-HT overflow (NET FR) was 1.9 ± 0.2 and 2.2 ± 0.1%. NST (1 μM) inhibited the K^+-evoked tritium overflow approximately to the same extent ($\sim$25%) in $NOP^{+/+}$ and $NOP^{-/-}$ mice. Conversely, N/OFQ inhibited tritium overflow in $NOP^{+/+}$ ($\sim$30%) but not $NOP^{-/-}$ mice.

To provide evidence for $G_{i/o}$ protein involvement in the b-NST inhibition of [^{3}H]-5-HT overflow, we tested maximally effective b-NST concentrations in synaptosomes pretreated with PTX (5 nM; Figure 4). For a comparison, the effect of N/OFQ under the same conditions was also investigated. PTX treatment did not modify the spontaneous tritium efflux (FR, 5.7 ± 0.1 and 5.9 ± 0.1% in the absence and presence of the toxin, respectively; $n=20$) nor the K^+-evoked [^{3}H]5-HT overflow (NET FR, 2.0 ± 0.1 and 1.8 ± 0.1%, respectively, in the absence and presence of PTX; $n=20$). However, in the presence of PTX, both N/OFQ (0.1 μM) and NST (1 μM) failed to inhibit the K^+-evoked [^{3}H]5-HT overflow (Figure 4).

Finally, experiments were carried out to investigate whether the two peptides could interact to modulate tritium overflow (Figure 5). First b-NST was challenged with N/OFQ following an 'agonist protocol'. Co-application of maximally effective concentrations of both peptides (1 and 0.1 μM, respectively), which produced similar inhibition of tritium overflow (Figure 1a), did not produce additive effects (Figure 5a). Then, we tested b-NST following an 'antagonist protocol'. Application of b-NST (1 μM) 9 min before N/OFQ (0.1 μM; that is, 18 min before K^+) significantly attenuated ($\sim$40%) N/OFQ inhibition (Figure 5b). Under these conditions, b-NST alone did not affect tritium overflow (see also Figure 1).

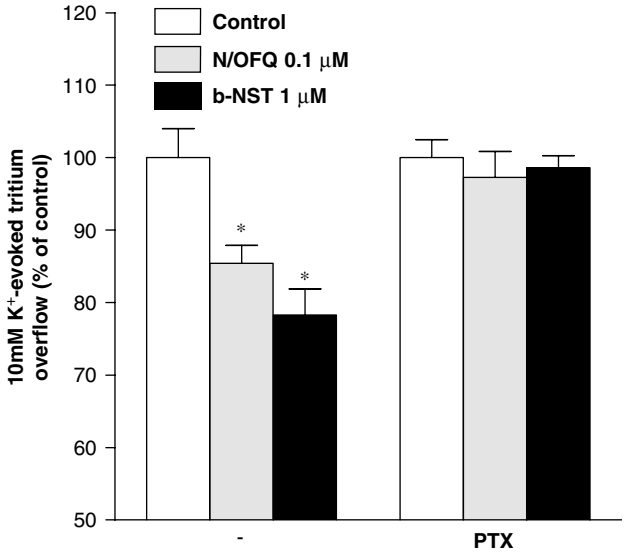

Figure 4 The effects of bovine nocistatin (b-NST) and nociceptin/orphanin FQ (N/OFQ) are prevented by *Pertussis* toxin (PTX). Effect of pretreatment with PTX (5 nM) on the inhibition of K^+-evoked [^{3}H]5-HT overflow induced by b-NST (1 μM) and N/OFQ (0.1 μM) in mouse neocortical synaptosomes. Data are expressed as percentage of the K^+ stimulation (control) and are means$\pm$s.e.m. of 8–12 determinations. *$P<0.05$; different from control (analysis of variance followed by the Newman–Keuls test for multiple comparisons).

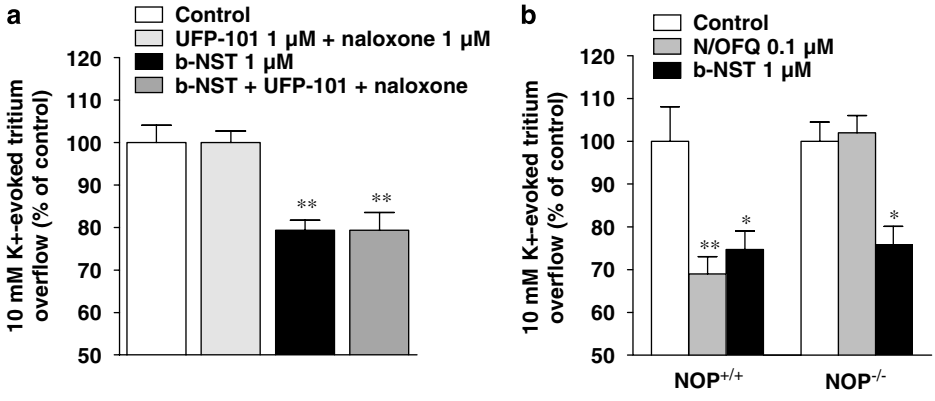

Figure 3 The effect of bovine nocistatin (b-NST) is mediated by neither classical opioid nor N/OFQ opioid peptide (NOP) receptors. (**a**) Effect of the non-selective opioid receptor antagonist naloxone (1 μM) and the NOP selective receptor antagonist [Nphe^1, Arg^{14}, Lys^{15}]N/OFQ(1–13)-NH_2 (UFP-101; 1 μM) on the inhibition of K^+-evoked [^{3}H]5-HT overflow induced by b-NST (1 μM) in neocortical mouse synaptosomes. Antagonists were perfused 3 min before b-NST and maintained until the end of the experiment. (**b**) Effect of b-NST (1 μM) and N/OFQ (0.1 μM) on the K^+-evoked [^{3}H]5-HT overflow from neocortical synaptosomes obtained from wild-type ($NOP^{+/+}$) and NOP receptor knockout ($NOP^{-/-}$) mice. Data are expressed as percentage of the K^+ stimulation (control) and are means$\pm$s.e.m. of 8–12 determinations. *$P<0.05$; **$P<0.01$, different from control (analysis of variance followed by the Newman–Keuls test for multiple comparisons).

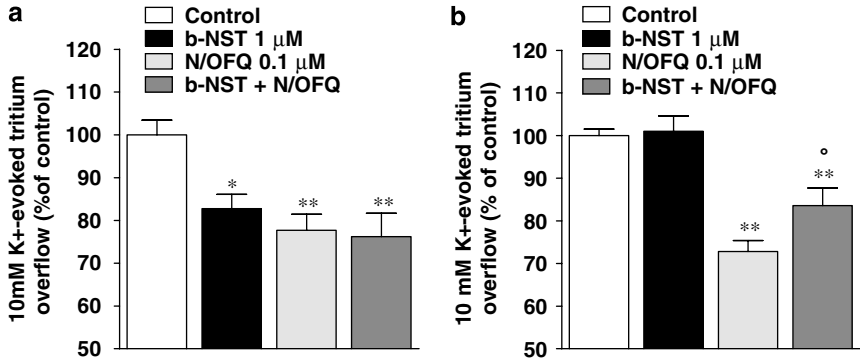

Figure 5 Nociceptin/orphanin FQ (N/OFQ) and bovine nocistatin (b-NST) interacted in modulating [^{3}H]5-HT overflow. Effect of co-application (**a**) or pre-application (**b**) of maximal b-NST (1 μM) and N/OFQ (0.1 μM) concentrations on the K^+-evoked [^{3}H]5-HT overflow. (**a**) Drugs were co-applied 9 min before K^+ and maintained until the end of the experiment. (**b**) b-NST and N/OFQ were applied 18 and 9 min before K^+, respectively. Data are expressed as percentage of the K^+ stimulation (control) and are means$\pm$s.e.m. of 12 (**a**) or 10 (**b**) determinations. *$P<0.05$, **$P<0.01$; different from control. °$P<0.05$; different from N/OFQ (analysis of variance followed by the Newman–Keuls test for multiple comparisons).

Discussion

NST is one of the products of cleavage of the prepro-N/OFQ precursor peptide, and it has been isolated from porcine, rat, mouse and human brain (Okuda-Ashitaka *et al*., 1998; Lee *et al*., 1999; Joseph *et al*., 2006). The original finding that NST antagonized N/OFQ actions, although not replicated in all the models tested (Connor *et al*., 1999; Habler *et al*., 1999; Shirasaka *et al*., 1999; Amano *et al*., 2000), was particularly attractive since it put forward the hypothesis that different by-products of the N/OFQ gene could modulate, in opposite ways, a specific biological function (Zeilhofer *et al*., 2000; Gavioli *et al*., 2002). A number of reports have pointed out a physiological role for NST in the regulation of pain transmission at the spinal cord level (for a review see Okuda-Ashitaka and Ito, 2000). However, consistent with the widespread expression of the N/OFQ gene in the CNS (Neal *et al*., 1999), evidence has been presented for an involvement of this peptide in the modulation of other central functions such as mood (Gavioli *et al*., 2002), learning and memory (Hiramatsu and Inoue, 1999) and food intake (Olszewski *et al*., 2000).

We previously showed that N/OFQ inhibited exocytotic (that is TTX- and Ca^{2+}-dependent) 5-HT overflow in the rat and mouse neocortex via activation of presynaptic NOP receptors (Sbrenna *et al*., 2000; Marti *et al*., 2003; Mela *et al*., 2004). Indeed, N/OFQ inhibition of 5-HT release from mouse synaptosomes orthogonally perfused (a preparation that minimizes indirect effects) was prevented by both NOP receptor antagonists (such as UFP-101) and deletion of the NOP receptor gene (Mela *et al*., 2004). The present finding that PTX prevented N/OFQ inhibition of the K^+-stimulated [^{3}H]5-HT release confirmed NOP receptor involvement, since the NOP receptor is coupled to $G_{i/o}$ proteins (Connor *et al*., 1996; Knoflach *et al*., 1996). From a methodological point of view, entrapping PTX into synaptosomes (Raiteri *et al*., 2000) led to rapid and complete inhibition of N/OFQ effects, without the need of prolonged incubation with the toxin. Consistent with a previous study on [^{3}H]5-HT release from synaptosomes (Sarhan and Fillion, 1999), PTX did not affect K^+-stimulated tritium overflow under these conditions. b-NST reproduced the effects of N/OFQ on 5-HT release, evoking a similar degree of inhibition in both rats and mice (while N/OFQ was more effective in rats) and being consistently less potent than N/OFQ. A few *in vitro* studies have dealt with the effects of NST at the cellular levels; in two of these, b-NST has been shown to exert primary agonist activity, namely inhibition of glycinergic and GABAergic transmission in dorsal horn interneurons (Zeilhofer *et al*., 2000) or induction of neurogenesis in hippocampal neurons in culture (Ring *et al*., 2006). In both studies, NST qualitatively replicated N/OFQ effects, although, in the spinal cord, N/OFQ and b-NST inhibited different populations of synapses (Zeilhofer *et al*., 2000). From a quantitative point of view, a $pEC_{50}\sim6$ has been calculated for b-NST in spinal cord slices (Zeilhofer *et al*., 2000), while effective b-NST concentrations in a preparation of hippocampal neurons in culture were found to be >1 μM (Ring *et al*., 2006). These values are similar to those found in mouse cortical synaptosomes but greater than those observed in the rat preparation, possibly suggesting the existence of species-dependent sensitivity to b-NST. Consistent with previous findings that b-NST did not displace [^{3}H]N/OFQ binding nor did it prevent N/OFQ inhibition of forskolin-induced cyclic AMP accumulation (Okuda-Ashitaka *et al*., 1998), b-NST inhibition was not attenuated by NOP receptor antagonists or deletion of the NOP receptor gene. Likewise, 1-[(3*R*,4*R*)-1-cyclooctylmethyl-3-hydroxymethyl-4-piperidyl]-3-ethyl-1,3-dihydro-2*H* benzimidazol-2-one (J-113397, a non-peptide NOP receptor antagonist) did not attenuate pro-nociceptive response to b-NST *in vivo* (Inoue *et al*., 2003) and deletion of the NOP receptor gene did not affect NST inhibition of neurotransmission *in vitro* (Ahmadi *et al*., 2001). b-NST effects in synaptosomes were also naloxone-insensitive, suggesting that the actions of b-NST were not mediated by either the classical opioid receptors or the NOP receptor. Nevertheless, consistent with reports that PTX abolished b-NST-evoked responses *in vivo* (Inoue *et al*., 2003) and *in vitro* (Zeilhofer *et al*., 2000), PTX also prevented b-NST inhibition of synaptosomal 5-HT release. Zeilhofer *et al*.

(2000) have proposed that a yet unidentified non-opioid and $G_{i/o}$ protein-coupled receptor mediates electrophysiological effects of NST in the spinal cord. Such a receptor may also be located on cortical nerve terminals releasing 5-HT, although the failure of b-NST in promoting GTP-γ-S binding in brain slices (Neal *et al*., 2003) challenges this view. However, the autoradiographic method employed in that study may not be sensitive enough to detect small changes at the presynaptic level. To confirm the presence of presynaptic NST–$G_{i/o}$ protein-linked pathways, loss of response following high b-NST concentrations or prolonged b-NST application time was detected. Consistent with a previous study (Mela *et al*., 2004), such a loss of response was also observed for N/OFQ. In that study, a 9-min exposure to high concentrations (10 μM) of N/OFQ or the NOP receptor peptide agonist [Arg^{14}, Lys^{15}]N/OFQ failed to affect [^{3}H]5-HT release. However, when applied for a shorter period (that is 3 min), N/OFQ (10 μM) maximally inhibited tritium overflow, suggesting that the loss of response was related to time- and concentration-dependent NOP receptor desensitization. In our preparation, b-NST and N/OFQ clearly activate different receptors although they both act through a $G_{i/o}$ protein-linked pathway. We thus can speculate that receptor desensitization also underlies the loss of response to high b-NST concentrations. Of note, the anxiogenic effect of b-NST *in vivo* was also described to follow a bell-shaped dose–response curve (characterized by loss of response to high doses; Gavioli *et al*., 2002). *In vitro*, no bell-shaped curve has been reported for b-NST so far. Instead, a careful concentration–response study performed in spinal cord slices showed no loss of response following concentrations as high as 10 μM b-NST or 100 μM rat NST (Zeilhofer *et al*., 2000). It is possible, however, that experimental reasons (for example, brain area investigated, loss of tissue architecture and/or receptor modulators, different kinetics of drug diffusion) can explain the discrepancy with our study.

On the basis of the positive cooperation between mu opioid (MOP) and NOP receptor agonists in regulating 5-HT release found in rat neocortical synaptosomes (Sbrenna *et al*., 2000), we attempted to investigate the reciprocal interactions between b-NST and N/OFQ in the same mouse preparation. The lack of additivity at maximal concentrations may be due to a ceiling effect (see also Sbrenna *et al*., 2000). Alternatively, the possibility of a different representation of NOP and (putative) NST receptors on different populations of serotonergic nerve terminals cannot be ruled out, since evidence that serotonergic nerve terminals are heterogeneous in terms of morphology and pharmacological properties has been presented (Kosofsky and Molliver, 1987; Fritschy *et al*., 1988; Brown and Molliver, 2000). This view, however, was challenged by the finding that prolonged exposure to b-NST attenuated responses to N/OFQ, which indicates that the two receptors colocalize to some extent. On the basis of this finding, it may be speculated that partial loss of N/OFQ inhibition after prolonged (minutes) exposure to b-NST reflects the occurrence of short-term heterologous desensitization, which has been described within the opioid receptor family (Kovoor *et al*., 1995; Samoriski and Gross, 2000).

Concluding remarks

Using a combined genetic and pharmacological approach, we demonstrated that b-NST inhibits 5-HT release in the mouse neocortex via activation of a presynaptic $G_{i/o}$ protein-mediated pathway not involving classical opioid receptors or the NOP receptor. These results represent the first direct evidence of the presence of a functional NST–$G_{i/o}$ protein-linked pathway on 5-HT terminals in the cerebral cortex and strengthen the view that NST can act through signal transduction mechanisms distinct from N/OFQ. It has been proposed (Calo *et al*., 2000) that the anxiolytic effect of N/OFQ (Jenck *et al*., 1997) is due to its ability to inhibit 5-HT transmission both at the somatodendritic (Vaughan and Christie, 1996) and nerve terminal (Sbrenna *et al*., 2000; Marti *et al*., 2003; Mela *et al*., 2004) level. Thus, based on the present data, one would expect NST to replicate the anxiolytic effects of N/OFQ. b-NST has been reported to modulate anxiety *in vivo* following a bell-shaped curve (Gavioli *et al*., 2002). Therefore, the possibility that the loss of response (observed at higher doses) is the result of recruitment of anxiolytic mechanisms opposing the anxiogenic ones (activated at lower doses) appears justified on the basis of the present results. In conclusion, although the physiological meaning of the present results remains to be investigated, this study indicates that NST modulates cortical 5-HT transmission and, therefore, may influence biological functions regulated by 5-HT, other than pain transmission.

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Conflict of interest

The authors state no conflict of interest.

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