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Investigation of the effects of nociceptin and nocistatin on pregnant uterine contractions in vitro

Ph.D. Thesis

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Appendix

Publications related to the Ph.D. thesis

I. Klukovits A, Tekes K, Gündüz Cinar O, Benyhe S, Borsodi A, **Deák BH**, Hajagos-Tóth J, Verli J, Falkay G, Gáspár R. Nociceptin inhibits uterine contractions in term-pregnant rats by signaling through multiple pathways. Biol Reprod 2010; 83:36-41. **IF:3.87**

II. **Deák BH**, Klukovits A, Tekes K, Ducza E, Falkay G, Gáspár R. Nocistatin inhibits pregnant rat uterine contractions in vitro: Roles of calcitonin gene-related peptide and calcium-dependent potassium channel. Eur J Pharmacol 2013; 714: 96-104. **IF:2.592**

III. **Deák BH**, Klukovits A, Kormányos Z, Tekes K, Ducza E, Gáspár R. Uterus-Relaxing Effects of Nociceptin and Nocistatin: Studies on Preterm and Term-Pregnant Human Myometrium In vitro. Reprod Sys Sexual Disorders 2013; 2:117.

Abstracts

I. **Deák Beáta**, Klukovits Anna, Kormányos Zsolt, Falkay György, Gáspár Róbert. A nociceptin és nocisztatin hatása a terhes humán és patkány uterusz kontraktilitására. A Magyar Élettani Társaság (MÉT) és a Magyar Kísérletes és Klinikai Farmakológiai Társaság (MFT) II. Közös tudományos konferenciája; Szeged, 2010. június 16-18.

II. **Beáta H Deák**, Anna Klukovits, Eszter Ducza, Zsolt Kormányos, Attila Pál, George Falkay, Róbert Gáspár. The signalling pathways of nociceptin and nocistatin in the pregnant human and rat uterine contractility. Pharmaceutical Sciences for the Future of Medicines Conference; Prague, Czech Republic, 13-17 June, 2011.

III. **Beáta H Deák**, Anna Klukovits, Eszter Ducza, Zsolt Kormányos, Attila Pál, George Falkay, Róbert Gáspár. The signalling pathways of nociceptin and nocistatin in the pregnant human and rat uterine contractility. Molekulától a gyógyszerig; Szeged, 2012. május 24-25.

List of abbreviations

NOP	nociceptin receptor
PNOC	prepronociceptin
N/OFQ	nociceptin/orphanin FQ
SP	substance P
CGRP	calcitonin gene-related peptide
cAMP:	cyclic adenosine monophosphate
NST:	nocistatin
G-protein:	heterotrimeric guanine-nucleotide binding regulatory protein
G_i:	inhibitory G-protein
G_s:	stimulatory G-protein
GTPγS:	guanosine-5'-O-(γ-thio)triphosphate
BK_{Ca}:	Ca^{2+}-dependent K^+ channels
IBMX:	3-isobutyl-1-methylxanthine
NX:	naloxone
$PGF_{2\alpha}$:	prostaglandine F2 alpha
PTX:	pertussis toxin
TEA:	tetraethylammonium
PAX:	paxilline
RIA:	radioimmunoassay
Real-time RT-PCR:	reverse transcription polymerase chain reaction
VMN:	ventromedial nucleus

1. Introduction

1.1. Causes and consequences of preterm birth and strategies for tocolytic therapy

Historically, the definition of prematurity was 2500 grams or less at birth. The current World Health Organization definition of prematurity is a baby born before 37 weeks of gestation, counting from the first day of the last menstrual period. Preterm birth occurs in 11% of live births globally and accounts for 35% of all newborn deaths. The rate is very high in Hungary too, it was 8.9 % of the total live births in 2010 (Fogarasi-Grenczer and Balázs, 2012). Preterm birth is a significant public health concern, as it is associated with high risk of infant mortality, various morbidities in both the neonatal period and later in life, and causes a significant societal economic burden (Ferguson *et al.*, 2013). Medical conditions such as anatomical disorder of the uterus, placenta praevia, chronic hypertension, diabetes, infections, preterm birth in previous pregnancy and stress are associated with preterm birth. Maternal age (younger than 16 or older than 40), low socio-economic status, drug or alcohol abuse, low maternal weight, racial and ethnic factors also play significant roles (Behrman *et al.*, 2007). There are also problems brought about by infertility treatment that often results in twins and triplets who are more likely born preterm. Nevertheless, in 50% of all cases the cause of preterm birth can not be identified.

Preterm-born babies are at higher risk for intraventricular bleeding, respiratory distress syndrome, bronchopulmonal dysplasia and necrotizing enterocolitis. Longer-term consequences of preterm birth may include respiratory, gastrointestinal problems, weak immune system, cerebral palsy, mental retardation, visual and hearing impairments, behaviour and social-emotional concerns, learning difficulties, and poor health and growth.

Uncertainty continues about the best strategies for managing preterm labor. Management of preterm delivery includes beta mimetics such as ritodrine and terbutaline; magnesium sulphate; prostaglandin inhibitors (for example, indomethacin and ketorolac); calcium channel blockers such as nifedipine; nitrates (for example, nitroglycerine); oxytocin receptor blockers (for example, atosiban). A recent pooled meta-analysis and decision analysis of trials on tocolytics showed that to delay delivery for 48 hours and seven days, prostaglandin inhibitors were the best tocolytics. Prostaglandin inhibitors and calcium channel blockers had the highest probability of delaying delivery and improving neonatal outcomes (Haas *et al.*, 2012). Bacterial vaginosis during pregnancy has been associated with poor perinatal outcomes and, in particular, preterm birth**.** Antibiotic treatment can eradicate bacterial vaginosis in

pregnancy, though the overall risk of preterm birth was not significantly reduced (Brocklehurst *et al.*, 2013).

As its underlying causes and molecular pathways have not been fully elucidated, there is a need for investigations of endogenous factors that might control uterine activity, with the perspective of improving tocolytic therapy.

1.2 Nociceptin/orphanin FQ and the nociceptin receptor (NOP): isolation, structure and the biological effects.

The peptide nociceptin/orphanin FQ (N/OFQ) was isolated for the first time in 1995 by two distinct research groups from rat and porcine brain (Meunier *et al.*, 1995; Reinscheid *et al.*, 1995). N/OFQ consists of seventeen amino acids. It was nominated nociceptin referring to the hyperalgesic effects that it evokes after supraspinal administration (Meunier *et al.*, 1995).

In parallel it was also called orphanin FQ, which name was given by Reinscheid *et al.* due to its high affinity of binding to the orphan opioid receptor (ORL1, hereafter referred to as NOP). They involved the first and last amino acids of the protein in its nomenclature: F and Q were used to sign the amino acids phenylalanine on the N-terminal (Phe, F) and glutamine (Gln, Q) located in the C-terminal position (Reinscheid *et al.*, 1995). According to the novel terminology it is commonly used as nociceptin/orphanin FQ (N/OFQ).

The nociceptin receptor (NOP) is similar in sequence to opioid receptors. Moreover, N/OFQ has a primary structure similar to that of opioid peptides. The synthetic heptadecapeptide potently inhibits forskolin-stimulated adenylate cyclase in CHO (ORL1+) cells in culture; this inhibitory activity being not affected by the addition of opioid ligands, nor did the peptide activate opioid receptors. When injected intracerebroventricularly into mice, orphanin FQ caused a decrease in locomotor activity but did not induce analgesia in the hot-plate test. In the rat *in vivo*, nociceptin diminishes acetylcholine release in the striatum, reduces dopamine release, and prevents the stimulatory effect of morphine on this transmitter in the nucleus accumbens and also elevates extracellular glutamate and γ-aminobutyric acid levels in mesencephalic dopaminergic areas. The effect of nociceptin on the mesencephalic dopaminergic system might explain its actions on motor behaviour (Schlickera *et al.*, 2000). However, the peptide produced hyperalgesia in the tail-flick assay. Thus, orphanin FQ may act as a transmitter in the brain by modulating nociceptive and locomotor behaviour (Reinscheid *et al.*, 1995). N/OFQ could modulate the outcome of some inflammatory diseases, such as sepsis and autoimmune pathologies by mechanisms not clearly elucidated yet. In fact, human body fluid revealed increased levels of N/OFQ under sepsis, arthritis, and

Parkinson's diagnose. The functional capacity of NOP receptor was demonstrated *in vitro* and *in vivo* studies by the ability of N/OFQ to induce chemotaxis of immune cells, to regulate the expression of cytokines and other inflammatory mediators, and to control cellular and humoral immunity (Gavioli *et al.*, 2011). The ventromedial nucleus (VMN) of the hypothalamus contains a population of neurons that inhibit feeding when they are active. The neuropeptide N/OFQ acts within the VMN to stimulate food intake. N/OFQ, through its receptor, directly inhibits these VMN neurons, thus by inhibiting anorexigenic VMN neurons, N/OFQ thereby allows the stimulation of food intake (Chee *et al.*, 2011). The peptide induces vasorelaxation of isolated rat arteries (Gumusel *et al.*, 1997) and inhibits contractions in isolated guinea-pig bronchus (Rizzi *et al.*, 1999), ileum (Zhan *et al.*, 1997), mouse vas deferens (Calo *et al.*, 1996; Zhang *et al.*, 1997) and rat bladder (Giuliani *et al.*, 1998). Furthermore, it potently contracts isolated rat (Taniguchi *et al.*, 1998) and mouse (Osinski *et al.*, 1999) colon. The changes of N/OFQ levels may play an important role in the pathophysiological changes in perinatal ischemia and hypoxia as well (Gu *et al.*, 2003). N/OFQ is essentially an anxiolytic peptide that plays a role in adapting to stress. N/OFQ counteracts the rewarding properties of abused drugs. N/OFQ has well-recognized diuretic actions that could explain increased fluid intake. The endogenous nociceptin system has a physiological role in mediating or regulating behavioral responses to alcohol, and that activating NOP receptors suppresses ongoing alcohol consumption or reinstatement of responding for alcohol. Alcohol-preferring rats acutely stimulated alcohol drinking, but produced a reversible suppression of drinking when administered repeatedly over seven days (Murphy *et al.*, 2010). Intrahippocampal administration of nociceptin was shown to produce a profound impairment of spatial learning, first in rats (Sandin *et al.*, 1997; Redrobe *et al.*, 2000) and later in mice (Kuzmin *et al.*, 2009). In all these tissues, the NOP receptor seems to be present in the peripheral endings of sensory, parasympathetic, and/or sympathetic nerves, where it exerts prejunctionnal modulation of mediator release. Moreover, under physiological conditions N/OFQ is present in the plasma of humans, in several pathological conditions such as female fibromyalgia syndrome (decreased level) (Anderberg *et al.*, 1998), postpartum depression (increased level) (Gu *et al.*, 2003), and it is a paracrine mediator of the FSH effects in the regulation of spermatogenesis (Eto *et al.*, 2012). N/OFQ also regulates the LH surge and ovarian function (Sinchak *et al.*, 2006).These results suggest that N/OFQ level changes may play a role in the function of the female and male reproductive tract.

1.3 *PNOC* gene and NOP receptor: structure and tissue distribution

It has been investigated that the *PNOC* gene transcript undergoes alternative splicing. So far, five splice variants of NOP receptor mRNA has been isolated. Except one slice variant that encodes a functional receptor which has been isolated from human tissue (Peluso *et al*., 1998), none of the other variants encode a full-length receptor (Mogil *et al.*, 2001), but rather non-functional truncated receptors. Both N/OFQ and its receptor share a high degree of sequence similarity to the opioid peptides and their corresponding receptors, respectively. However, N/OFQ does not activate opioid receptors, nor do the opioid peptides elicit biological activity at the NOP receptor (Reinscheid *et al.*, 1998). There are structural similarities between the amino acid sequences of N/OFQ and dynorphin A.

The NOP receptor is widely expressed in the central nervous system, particularly in the forebrain (cortical areas, olfactory regions, limbic structures, thalamus), throughout the brainstem (central periaqueductal gray, substantia nigra, several sensory and motor nuclei), and in both the dorsal and ventral horns of the spinal cord. Regions almost devoid of NOP receptors are the caudate-putamen and the cerebellum. The anatomic distribution of the NOP receptor also reveals that pharmacological effects, other than those on which many studies have already focused, are worthwhile to explore. Aside from the nervous system, the immune system is one of the principal locations of the NOP receptor. The NOP receptor was identified in the peripheral nervous system and several isolated organs. RT-PCR results indicate that the mRNA of NOP receptor is expressed in rat intestine and vas deferens (Wang *et al.*, 1994) as well as porcine gastrointestinal tract and kidney (Osinski *et al*., 1999). NOP transcripts were also detected in several guinea-pig ganglia (Fischer *et al.*, 1998; Kummer *et al.*, 1997). Finally, high affinity binding sites for N/OFQ were detected in the retina (Makman *et al.*, 1997) and the heart (Dumont *et al.*, 1998) of the rat. In the periphery N/OFQ precursor mRNA was also detected in human spleen, peripheral blood leukocytes, in the rat ovary and fetal kidney (Mollereau *et al*., 1996; Nothacker *et al*., 1996).

The PNOC gene is highly conserved in the three species (human, mouse and rat) and displays organizational features that are strikingly similar to those of the genes of preproenkephalin, preprodynorphin, and preproopiomelanocortin, the precursors to endogenous opioid peptides, suggesting the four genes belong to the same family-i.e., have a common evolutionary origin. PNOC is also weakly expressed in the ovary. The presence in ovary and hypothalamus suggests that the N/OFQ system may also play a role in endocrine regulation (Mollereau *et*

al., 1996). However, the possible presence and actions of N/OFQ on the smooth muscle of the female reproductive organs have not been discussed.

1.4 Nocistatin: structure and effects

Okuda-Ashitaka isolated for the first time the other biologically active neuropeptide derived from the same precursor as N/OFQ (Okuda-Ashitaka *et al.*, 1998). The endogenous heptadecapeptide nocistatin (NST) was isolated from bovine brains and recently identified in mouse, rat, and human brain and in human cerebrospinal fluid. Although human, rat and mouse NST produced larger respective counterparts with 30, 35, and 41 amino acid residues, all peptides showed the antinociceptive activity. This activity was ascribed to the carboxyl-terminal hexapeptide Glu-Gln-Lys-Gln-Leu-Gln, which is conserved beyond species. Intrathecal administration of N/OFQ induced pain responses including allodynia and hyperalgesia. Simultaneous administration of NST blocked the allodynia and hyperalgesia whereas anti-NST antibody decreased the threshold for the N/OFQ-induced allodynia. NST also attenuated the allodynia and hyperalgesia evoked by prostaglandin E2 (PGE_2) and the inflammatory hyperalgesia induced by formalin or carrageenan/kaolin, and reversed the N/OFQ-induced inhibition of morphine analgesia at picogram doses (Minami *et al.*, 1994a, 1994b, Taiwo and Levine, 1988 and Okuda-Ashitaka *et al.*, 1998). Furthermore, NST counteracted the impairment of learning and memory induced by N/OFQ or scopolamine (Hiramatsu and Inoue, 1999). NST suppresses appetite (Olszewski *et al.*, 2000) and induces gastric mucosal protection (Zádori *et al.*, 2008). It has been demonstrated that NST binds to a binding site that is distinct from the NOP receptor (Okuda-Ashitaka and Ito, 2000 and Johnson and Connor, 2007). Fantin *et al.* also showed that the putative receptor of NST is probably a G protein–coupled one. NST inhibits 5-hydroxytryptamine release via a $G_{i/o}$ protein-mediated pathway (Johnson and Connor, 2007 and Fantin *et al.*, 2007).

It has been demonstrated that chronic constriction injury and diabetic pain elicit specific significant increases in the levels of PNOC, N/OFQ and NST (Liu *et al.*, 2012).

Given the myorelaxant actions of opioids, these endometrial neuropeptides may participate in the control of myometrial contractility.

2. Aims

The main focus of our study was to investigate the myometrial effect of the endogenous neuropeptides N/OFQ and NST which are physiologically present in the humans and rats, and additionally have a role in the pain transmission during delivery. Therefore the following aims were set:

1. The main aim of the study was to investigate the roles and mechanisms of N/OFQ and NST in the uterine contractility in term-pregnant rat (day 22 of pregnancy) and in human myometrium obtained from full-term pregnancy and from preterm birth.

2. We set out to investigate the effect of N/OFQ and NST on the uterine contractility in an isolated organ bath system *in vitro,* by using term-pregnant rat uterine samples and myometrial samples obtained from caesarean sections of term-pregnant women, and from preterm delivery. Our further aim was to test the expression of N/OFQ and NST in the rat myometrium with radioimmunoassay and to detect the presence of their common precursor *PNOC* mRNA with real-time PCR technique.

3. The following aim was to find out the signalling pathways of N/OFQ and NST in the uterus with the means of [^{35}S]GTPγS binding assay (for G protein activation), enzyme immunoassay (cAMP accumulation) and in vitro contractility studies. We set out to investigate the possible role of the Ca^{2+}-dependent (outward rectifying) K^+ channels (BK_{Ca}) and the release of the sensory neuropeptide CGRP in the mechanism of action of N/OFQ and NST, as well as the influence of a hypocalcemic environment in the uterus-relaxant effect of NST in the presence of NX.

3. Materials and methods

3.1. Animals

The animals were treated in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.32.§). All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: IV./01758-2/2008) and under the control of the ISO-9001:2008 Quality Management System.
Sexually mature female Sprague-Dawley rats (body mass: 160-200 g, 50-60 days old) were mated in the early morning hours. Copulation was confirmed by the presence of a copulation plug or spermatozoa in the vagina. The day of copulation was considered to be the first day of pregnancy. The animals were housed in temperature (20-23 °C), humidity (40-60%) and light (12 h of light, 12 h of dark)-regulated rooms, with water and food intake available *ad libitum*.

3.2. Human specimens

Biopsy specimens of human myometrial tissue were obtained at cesarean section (performed in the Department of Obstetrics and Gynecology, University of Szeged) in the third trimester of pregnancy in two groups: at full-term birth (37-41 weeks of gestation; n=10) and at preterm birth (33-36 weeks; n=9). At full-term, cesarean delivery was indicated by a previous cesarean delivery, breech presentation, a suspected cephalopelvic disproportion or myopia. The parity varied from 0 to 3, and the mean maternal age was 28.4 years (21-35 years). None of the women received a tocolytic agent, and there were no signs of labour.
Preterm delivery occurred in mothers with twin pregnancies, or labour was indicated by an ongoing infection, leukocytosis, toxaemia, foetal distress or growth restriction. In the preterm group, the parity varied from 0 to 3, and the mean maternal age was 28.2 years (18-38 years). Three of the 9 patients received tocolytic therapy (magnesium sulphate) to arrest preterm uterine contractions, which proved to be ineffective. All the operations were performed under spinal anaesthesia. The Ethical Committee of Albert Szent-Györgyi Clinical Centre, University of Szeged, approved the clinical protocol for the use of human tissue; the pregnant women signed an informed consent form, uninfluenced (registration number: 114/2009).

3.3. Real-time RT-PCR (reverse transcription polymerase chain reaction) studies

3.3.1. Rat study: On selected days of late pregnancy (days 18, 20 and 22), rats were killed by CO_2 inhalation, the uteri were excised and trimmed of fat, the feto-placental units were removed and the endometrium was denuded. The tissue samples were frozen immediately in liquid nitrogen, and then stored at -70 °C until analysis. The frozen samples were ground with a Micro-Dismembrator S homogenizer (Sartorius, Germany), and the total RNA was isolated with the TRIsure Kit according to the manufacturer's instructions. RNA purity was controlled via the optical density at 260/280 nm with a BioSpec Nano instrument (Shimadzu, Japan); all samples exhibited an absorbance ratio in the range 1.6-2.0. RNA quality and integrity were assessed by agarose gel electrophoresis. One microgram of each sample of total RNA was used for reverse transcription and amplification (TaqMan RNA-to-C_T 1-Step Kit and the Sensi FAST Probe Hi-Rox One-Step Kit). The following primers were used: assay ID Rn01637101_m1 for *PNOC,* Rn 00667869_m1 for *ß-actin* and Rn 01775763_g1 for *GAPDH* as endogenous controls. RT-PCR was performed by using the ABI StepOne Real-Time cycler. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant fluorescence signal increase was defined as the threshold cycle (C_T).

There is an ongoing debate about the applicability of beta-actin as a gene reference in the pregnant rat uterus, with no reassuring conclusion as yet (Kelly *et al.*, 2003). Thus we have repeated our PCR studies also with GAPDH as a gene reference. We did not find differences in the change of *PNOC* mRNA when GAPDH was used as compared with beta-actin.

3.3.2. Human tissue collection: The human uterus tissue samples were frozen in liquid nitrogen, and then stored at -70 °C until analysis. The frozen samples were ground with a Micro-Dismembrator S homogenizer (Sartorius, Germany), and the total RNA was isolated with the TRIsure Kit according to the manufacturer's instructions. RNA purity was controlled via the optical density at 260/280 nm with a BioSpec Nano instrument (Shimadzu, Japan); all samples exhibited an absorbance ratio in the range 1.6-2.0. RNA quality and integrity were assessed by agarose gel electrophoresis. One microgram of each sample of total RNA was used for reverse transcription and amplification (TaqMan RNA-to-C_T 1-Step Kit and the Sensi FAST Probe Hi-Rox One-Step Kit).

The following primers were used: assay ID Hs00173823_m1 for *PNOC* and Hs 01060665_g1 for *ß-actin* as endogenous control. RT-PCR was performed by using the ABI StepOne Real-Time cycler. The fluorescence intensities of the probes were plotted against PCR cycle numbers. The amplification cycle displaying the first significant fluorescence signal increase was defined as the threshold cycle (C_T).

3.4. Radioimmunoassay (RIA) for N/OFQ and NST in the rat uterus

The uterine levels of N/OFQ were measured in nonpregnant and 22-day pregnant rats and the levels of NST were evaluated in nonpregnant and 15-, 18-, 20- and 22-day pregnant rats. The extraction of N/OFQ and NST was carried out by a validated method (Tekes *et al.*, 2005; Eun-Mee *et al.*, 1999 and Hofbauer *et al.*, 2000). Uteri were treated with 1.0 ml of 1 M acetic acid at 95 °C for 5 min, then cooled in ice-cold water bath for 10 min, then homogenized by an Ultra Turrax T25 Janke&Kunkel homogenizer at 20,000 rpm/min for 10 sec (IKA Labortechnik, Staufen, Germany), followed by sonication (Labsonic 2000, B. Braun AG, Melsungen, Germany) for 30 sec. Homogenates were repeatedly placed for 5 min in a 95 °C shaking water bath, then left to sediment for 10 min at 0 °C, and centrifuged in an Eppendorf centrifuge (A. Hettich, Tuttlingen, Germany) at 14,000 g for 10 min at 4 °C. 1.0 ml aliquots of the supernatants were mixed with an equal volume of 1% v/v trifluoroacetic acid (TFA I), loaded onto C18 Sep-Pack cartridges (ABL&E Jasco Hungary Ltd., Budapest), washed twice with TFA I, and then eluted with 60% acetonitrile in 0.1% TFA. Samples were freeze-dried by centrifugation (Savant Instruments Inc., Farmingdale, NY, USA).

During the validation of the extraction procedure, the stability of N/OFQ as well as the reproducibility, recovery, and linearity of recovery in the concentration range of 1–100 pg/100 mg tissue were determined. The recovery was 87% ± 0.68% (mean ± SEM), and all other parameters were in the normal ranges. During the validation of the extraction procedure stability of NST, reproducibility, recovery and linearity of recovery in the 10–500 pg/100 mg tissue concentration range were determined. Recovery was 89 ± 0.87 %, and all the other parameters were in the normal range.

The tissue extracts were subjected to radioimmunoassay (RIA) for N/OFQ using a commercially available ^{125}I-N/OFQ RIA Kit (Nociceptin/Orphanin FQ [rat] - RIA Kit; Phoenix Pharmaceuticals Inc.) with a minimum sensitivity of 1 pg/ml and for NST using a commercially available ^{125}I-Nocistatin RIA kit with minimum sensitivity of 10 pg/ml. Data were evaluated with Isodata 20/20 software (Isodata, Pittsburg, KS, USA) and by RIA-Mat 280 (Byk-Sangtec, Dietzenbach, Germany). Significance was calculated by Student *t*-test.

3.5. Radioligand-binding experiments

3.5.1. Membrane preparation

Radioligand-binding experiments were carried out on nonpregnant or 22-day-pregnant rat uterus membrane preparations. The uterine tissues were cut and homogenized in ice-cold buffer (0.05 M Tris-HCl, pH 7.4) using a Braun teflon-glass homogenizer (10-15 strokes), and filtered through four layers of gauze to remove large aggregates. The homogenate was centrifuged (Sorvall RC5C centrifuge, SS34 rotor) at 40,000 *g* for 20 min at 4 °C, and the resulting pellet was resuspended in fresh buffer and incubated for 30 min at 37 °C. The centrifugation step was repeated, and the final pellet was resuspended in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose and stored at –70 °C until use (Ligeti *et al.*, 2005). Protein concentration was determined by the method of Bradford (Bradford *et al.*, 1976).

3.5.2. Radioligand-binding assay

For the homologue displacement experiments, the reaction mixture contained 100 µl of membrane preparation (~ 0.3-0.4 mg protein/ml), 100 µl of [^{3}H]N/OFQ-NH_2 with a specific activity of 25 Ci/mM, and 100 µl of unlabelled N/OFQ (10^{-12}–10^{-5} M) or 100 µl of incubation buffer (consisting of 0.05 M Tris-HCl, 0.01 M $MgCl_2$ and 2.5% ethanol, pH 7.42) for total binding. Incubation was started by addition of the membrane suspension, and continued in a shaking water bath until a steady state was achieved (24 °C, 60 min). At the end of the incubation, the bound radioligand was separated from the residual free radioligand by rapid filtration on a Brandel M24R Cell harvester (Semat, UK) through Whatman GF/C filters (Semat Technical Ltd, St. Albans UK) pre-soaked in polyethyleneimine (0.3%, pH 10) for 30 min and washed with 3 x 10 ml of ice-cold buffer (0.05 M Tris-HCl, pH 7.42). The radioactivity of the dried filters was detected in UltimaGold^{TM} F scintillation cocktail (Packard) with a Packard Tricarb 2300TR liquid scintillation counter (Ligeti *et al.*, 2005).
Specific binding was determined by subtracting the non-specific binding value from the total binding value. All assays were carried out at least 3 times in duplicate and values are given as the mean ± S.E.M. The experiments were individually analyzed and the maximum numbers of binding sites (B_{max}) and the equilibrium dissociation constants (K_d) were calculated.

3.5.3. [^{35}S]GTPγS-binding assay

The uterine tissue samples obtained from 22-day-pregnant rats were homogenized (Gündüz *et al.*, 2006) and diluted in 50 mM Tris-HCl buffer (pH 7.4) to obtain appropriate protein content for the assays (~ 10 μg of protein/sample). The membrane fractions were incubated at 30 °C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, and containing 20 MBq/0.05 ml of [^{35}S]GTPγS (0.05 nM) and increasing concentrations of N/OFQ tested in the presence of excess GDP (30 μM) in a final volume of 1 ml, according to Sim *et al.* and Traynor and Nahorski (Sim *et al.*, 1995; Traynor and Nahorski, 1995), with slight modifications. The effect of N/OFQ was investigated together with 10^{-6} M NX. The G_i-protein-activating effect of N/OFQ and NX was also measured in the presence of 500 ng pertussis toxin (PTX)/ml. Total binding (T) was measured in the absence of test compound; non-specific binding (NS) was determined in the presence of 10 μM unlabelled GTPγS and subtracted from T. The difference (T–NS) indicates the basal activity. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters (Whatman, Dassel, Germany) with a Brandel M24R Cell harvester. Filters were washed three times with 5 ml of ice-cold buffer (pH 7.4), and the radioactivity of the dried filters was detected in UltimaGold^{TM} F scintillation cocktail (Packard) with a Packard Tricarb 2300TR liquid scintillation counter. Stimulation is given as a percentage of the specific [^{35}S]GTPγS-binding observed in the absence of receptor ligands (basal activity). The [^{35}S]GTPγS-binding experiments were performed in triplicate and repeated at least three times. Data were analysed with the sigmoid dose–response curve fit option.

3.6. Measurement of uterine cAMP accumulation

As cAMP elevation plays a major role in the relaxation of uterine smooth muscle, we set out to investigate its possible involvement in the N/OFQ and NST signalling. Uterine tissue samples were incubated in de Jongh solution at 37 °C, perfused with carbogen. cAMP accumulation was determined in the presence of the non-specific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 10^{-3} M; 10 min), OFQ alone (10^{-8} M; 10 min) or in combination with NX, NST alone (10^{-8} M; 10 min) or in combination with N/OFQ or NX, and the adenylyl cyclase activator forskolin (10^{-5} M; 10 min). The effect of the G_i/G_o protein inhibitor PTX (500 ng/ml) on uterine cAMP accumulation was also tested in the presence of IBMX, N/OFQ, NX and forskolin. The samples were then immediately frozen in liquid nitrogen and stored until the extraction of cAMP. Frozen tissue samples were ground,

weighed, homogenized in 10 volumes of ice-cold 5% trichloroacetic acid and centrifuged at 1000 *g* for 10 min. The supernatants were extracted with 3 volumes of water-saturated diethyl ether. After drying, the extracts were stored at -70 °C until the cAMP assay. Uterine cAMP accumulation was measured with a commercial competitive cAMP enzyme immunoassay (EIA) kit and tissue cAMP levels were expressed in pmol/mg tissue. All samples (n=4 in each group) were measured in duplicate in the EIA.

3.7 *In vitro* contractility studies

3.7.1. Rat uterus preparation

On day 22 of pregnancy (at term), the rats were killed by CO_2 inhalation, and the uteri were removed and prepared for the in vitro contractility assay (Klukovits *et al.*, 2010). Briefly, the isolated uterine horns were placed immediately in an organ bath (de Jongh solution; containing in mM: 137 NaCl, 3 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 12 $NaHCO_3$, 4 Na_2HPO_4, 6 glucose; pH 7.4*)* perfused with a mixture of 95% oxygen and 5% carbon dioxide (carbogen) and trimmed of fat, and the foeto-placental units were removed. The temperature was maintained at 37 °C. Four rings 0.5 cm long were sliced from the middle part of each horn, including implantation sites, and tested in parallel; they were mounted vertically in the above-mentioned organ bath containing 10 ml of de Jongh solution. After mounting, the initial tension was set at 1.5 g and the rings were equilibrated for 60 min, with a solution change every 15 min.

3.7.2. Nociceptin studies in rat uterine tissues

In the isolated uterine rings, rhythmic contractions were elicited with 25 mM KCl.

The effects of N/OFQ and/or NX on the uterine rings were measured in the concentration range of 10^{-12} – 10^{-7} M in a non-cumulative manner. After each concentration of N/OFQ or NX, the rings were washed 3 times, allowed to recover for 5 min, and then contracted again with KCl.

In another set of experiments, uterine contractions were elicited with 10^{-8} M oxytocin and the contraction-inhibiting effects of N/OFQ alone and in combination with NX were tested in a non-cumulative manner. These experiments were carried out in the presence of the non-selective K^+ channel blocker tetraethylammonium (TEA; 10^{-3} M) and the BK_{Ca}-channel selective blocker paxilline (PAX; $5x10^{-6}$ M).

3.7.3. Nocistatin studies in rat uterine tissues

In the isolated uterine rings, rhythmic contractions were elicited with 25 mM KCl or with 10 nM oxytocin or with 1 μM $PGF_{2\alpha}$. Without washing out the contractile agents, the effects of NST on the uterine contractions were tested in the concentration range 10^{-12} – 10^{-6} M, in a noncumulative manner (as regards its peptide characteristic). After each concentration of NST, the rings were washed 3 times, allowed to recover for 5 min, and then contracted again with the above-mentioned agents. Following the oxytocin- and $PGF_{2\alpha}$-induced contractions, the contraction-inhibitory effect of NST was also investigated in the presence of N/OFQ (10^{-8} M).

Following the KCl-induced contractions, the contraction-inhibitory effect of NST was investigated in the presence of N/OFQ (10^{-8} M) and/or NX (10^{-8} M). The most potent inhibitory effect of NST was found in the KCl-induced contractions; this agent was therefore used to investigate the possible mechanism of NST in further studies. As NX induces an increase in inward Ca^{2+} currents (Kai *et al.*, 2002), we conducted a series of experiments in a hypocalcemic environment in order to investigate whether the inhibitory effect of NX on the NST-induced uterus relaxation is mediated by the opening of inward rectifying Ca^{2+} channels. Thus the joint effect of NST and NX was studied in a modified de Jongh buffer, containing half the Ca^{2+} concentration (0.5 mM $CaCl_2$) of the standard de Jongh buffer (Hajagos-Tóth *et al.*, 2009). In order to investigate the participation of the outward rectifying K^+ channels in mediating the effects of NST, tests were performed in the presence of the BK_{Ca} channel-selective blocker PAX ($5x10^{-6}$ M), against spontaneous uterine contractions.

The possible involvement of the sensory neuropeptide CGRP in the actions of NST was also tested on uterine tissue. In this set of experiments, capsaicin (1 μM dissolved in physiological saline containing 6% Tween 80 and 8% ethanol; for 10 min) was used to deplete CGRP from the uterine sensory nerve endings (Holzer *et al.*, 1991). After thorough washing out, the tissues were incubated with CGRP (0.1 μM; 20 min) (Sams-Nielsen *et al.*, 2001**)** and washed again, and the effects of NST were tested as above. These experiments were performed in a de Jongh solution supplemented with protease inhibitors such as phenylmethanesulfonyl fluoride (1 μM), captopril (0.1 mM), dithiothreitol (0.5 mM), soy bean trypsin inhibitor (1 mM) and aprotinin (36,000 KIU/L).

The tension of the myometrial rings was measured with a strain gauge transducer (SG-02, Experimetria Ltd, Budapest, Hungary), and recorded and analyzed with the SPEL

Advanced ISOSYS Data Acquisition System (Experimetria Ltd, Budapest, Hungary). The areas under the curves of 4-min periods were evaluated; the effects of NST, N/OFQ and NX were expressed as percentages of KCl/oxytocin/$PGF_{2\alpha}$-induced or spontaneous contractions. The concentration-response curves were fitted and the geometrical mean of $logEC_{50}$ values and maximum contraction-inhibitory values were calculated with the Prism 4.0 computer program (GraphPad Inc., San Diego, CA, USA).

3.7.4. Human uterus preparation

Each tissue sample (~10 × 10 × 30 mm) was obtained from the upper edge of a lower-segment transverse incision, after delivery of the child, but before oxytocin was given to the mother. Tissues were stored in Krebs–Henseleit solution (containing in mM: 118 NaCl, 5 KCl, 2 $CaCl_2$, 0.5 $MgSO_4$, 25 $NaHCO_3$, 1 $KHPO_4$, 10 glucose; pH 7.4) at 4 °C, until investigation, but within 12 hours of collection.
Longitudinal myometrial strips (~3 × 5 × 10 mm) were mounted vertically in an organ bath containing 10 ml Krebs–Henseleit solution. The organ bath was maintained at 37 °C, and carbogen (95% O_2 + 5% CO_2) was bubbled through it. After mounting, the initial tension was set at 3.0 g and the rings were equilibrated for 90 min, with a solution change every 15 min.

3.7.5. In vitro studies in human uterine tissues from full-term births and from preterm births

In the isolated uterine rings, rhythmic contractions were elicited with 10^{-8} M oxytocin. The effects of N/OFQ and NST on the uterine contractions were tested in the concentration range 10^{-12} – 10^{-6} M, in a non-cumulative manner. After each concentration of N/OFQ and NST, the rings were washed 3 times, allowed to recover for 5 min, and then contracted again with oxytocin. The uterus-relaxant effect of NST was also investigated in the presence of N/OFQ. Additionally, the uterus-relaxant effect of N/OFQ was investigated in the presence of NST.

The tension of the myometrial rings was measured with a strain gauge transducer (SG-02, Experimetria Ltd, Budapest, Hungary), and recorded and analyzed with the SPEL Advanced ISOSYS Data Acquisition System (Experimetria Ltd, Budapest, Hungary). The areas under the curves of 6-min periods were evaluated; the effects of NST and N/OFQ were expressed as percentages of the oxytocin-induced contractions.

3.8 Chemicals

The radioligand ([^{3}H]N/OFQ) was radiolabelled by Drs Judit Farkas and Geza Toth (Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary). The ^{125}I-Nociceptin Radioimmunoassay Kit was obtained from Phoenix Pharmaceuticals Inc., Belmont, CA, USA. Guanosine-5'-[γ-^{35}S]-triphosphate ([^{35}S]GTPγS) was purchased from Amersham, UK. PTX was from Tocris Cookson Ltd., Avonmouth, UK. N/OFQ and NST were purchased from PolyPeptide Laboratories France SAS, Strasbourg, France. $PGF_{2\alpha}$, NX, forskolin, IBMX, cAMP Enzyme Immunoassay Kit, TEA, PAX, guanosine 5'-diphosphate (GDP), guanosine-5'-o-(3-thiotriphosphate) (GTPγS), polyethyleneimine, capsaicin, soy bean trypsin inhibitor, dithiothreitol, phenylmethanesulfonyl fluoride and captopril were from Sigma-Aldrich Ltd, Budapest, Hungary. The TRIsure Kit and the Sensi FAST Probe Hi-Rox One-Step Kit was from Bioline Ltd. Budapest, Hungary. TaqMan RNA-to-C_T 1-Step Kit, ß-actin and GAPDH primers were obtained from Life Technologies, Budapest, Hungary. The ^{125}I-Nocistatin RIA kit was from Phoenix Pharmaceuticals, Inc., purchased by Izinta Ltd., Budapest, Hungary. Aprotinin (Gordox®) and oxytocin were purchased from Richter-Gedeon Ltd. Budapest, Hungary.

3.9 Statistical analyses

Statistical analyses were carried out by ANOVA Newman-Keuls multiple comparison test with the Prism 4.0 computer program (GraphPad Inc., San Diego, CA, USA). This test makes pairwise comparisons of group means. The alpha level of Newman -Keuls test is 0.05.

4. Results

4.1. Investigations on 22-day pregnant rat uterus

4.1.1. N/OFQ and NST tissue levels

The tissue levels of N/OFQ were measured by RIA in the uteri of non-pregnant and 22-day pregnant rats (n=5 for each group), and the myometrial NST levels were measured in the uteri of non-pregnant and 15, 18, 20, and 22-day pregnant rats respectively (n=6 for each group). In the 22-day pregnant rats, uterine N/OFQ concentration was significantly higher ($P<0.05$) than in the non-pregnant rats. The myometrial NST levels increased significantly as term was approached. The RIA experiments revealed that the levels of NST were relatively low on pregnancy days 15, 18 and 20 ($P>0.05$), then elevated significantly by day 22, the day of delivery; $P<0.05$ as compared with day 20. The NST levels on day 22 did not differ from the non-pregnant samples (**Table 1**).

Tissue	**N/OFQ**		**NST**	
	pg/100 mg uterine tissue ± S.E.M		**pg/100 mg uterine tissue ± S.E.M**	
Non-pregnant	2.11 ± 0.21		47.07 ± 4.66	
15 day pregnant			17.49 ± 4.41	*****
18 day pregnant			17.15 ± 3.03	**ns**
20 day pregnant			13.95 ± 1.82	**ns**
22 day pregnant	3.65 ± 0.12	*****	42.11 ± 6.27	*****

Table 1. Tissue N/OFQ and NST levels in non-pregnant, 15, 18, 20 and 22 day pregnant rats. *P<0.05, ns: nonsignificant. Significances are expressed relative to the value of the previous tested day.

4.1.2. Radioligand-binding studies

The presence of NOP receptors in the uterus was detected by radioligand-binding experiments (n=4 for each group). In the uteri of nonpregnant females, the maximum binding capacity (B_{max}) of the NOP receptors was 87.3 ± 5.2 fmol protein/mg membrane, with a dissociation constant (K_d) of 2.19 ± 0.14 × 10^{-8} M. In the membrane fractions of the 22-day-pregnant uteri, the corresponding B_{max} and K_d values were 99.6 ± 2.31 fmol protein/mg membrane and 1.95 ± 0.09 × 10^{-8} M, respectively. No significant difference ($P>0.05$) was found between the B_{max} or K_d values in the nonpregnant versus the 22-day pregnant rat uteri.

4.1.3. [^{35}S]GTPγS-binding assay

Because the central effects of N/OFQ are mediated via the activation of G_i proteins coupled to the NOP receptors and also the outward rectifying K^+ channels, the N/OFQ-stimulated G protein activation was additionally tested on the membrane fractions of the 22-day-pregnant rat uteri (**Fig. 1**). N/OFQ stimulated the [^{35}S]GTPγS binding through the NOP receptors by 119.1% ± 1.2%. In the presence of NX, the N/OFQ-stimulated G protein activation was decreased to 106.8% ± 1.2%. In the presence of PTX and NX, however, the maximum G protein activation decreased to 82.3% ± 3.0% with the activation declining to below the basal level (n=6 for each set of experiments; **Table 2**).

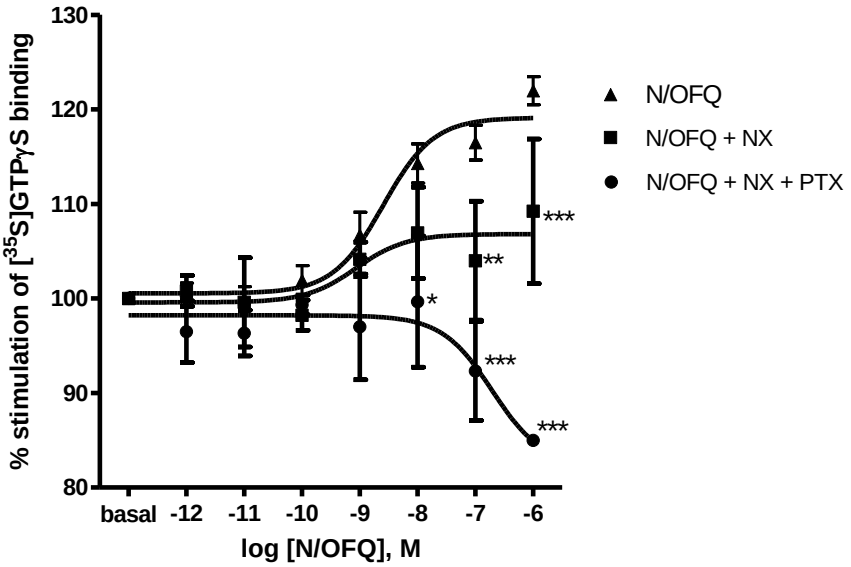


Fig. 1. Effect of nociceptin (N/OFQ) on G protein activation in the pregnant rat myometrium in vitro. N/OFQ increased the concentration of activated G protein in a dose-dependent manner (▲). The presence of 10^{-6} M naloxone (NX) significantly decreased the nociceptin-induced G protein activation (■), while the G_i protein inhibitor pertussis toxin (PTX) elicited a strong decline in activated G protein level (●). n=6; *P<0.05, **P<0.01, ***P<0.001.

	logEC_{50} (± S.E.M.)	**E_{max}** (% ± S.E.M.)
N/OFQ	-8.59 ± 0.17	119.1 ± 1.19
N/OFQ+NX	-8.35 ± 0.50	106.8 ± 1.26 ***
N/OFQ+NX+PTX	-6.71 ± 0.29	82.35 ± 3.04 ***

Table 2. logEC_{50} and maximum inhibitory values of nociceptin (N/OFQ) alone and in the presence of naloxone (NX) and of NX with pertussis toxin (PTX) on G protein activation in 22-day-pregnant uterine rings from the rat (n=6); ***P<0.001; significances are expressed relative to N/OFQ.

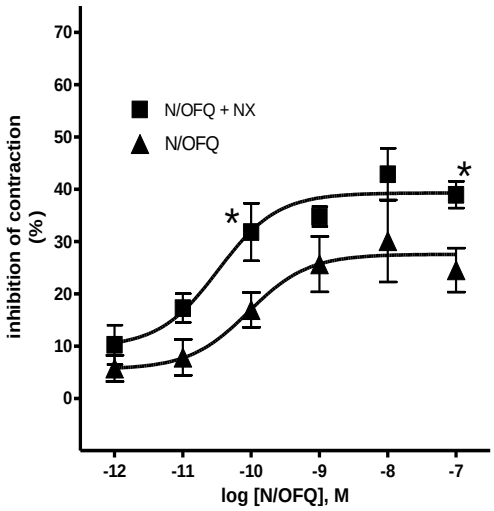
4.1.4. *In vitro* contractility studies

4.1.4.1. Investigation of the contraction-inhibiting effects of N/OFQ, NST, and NX

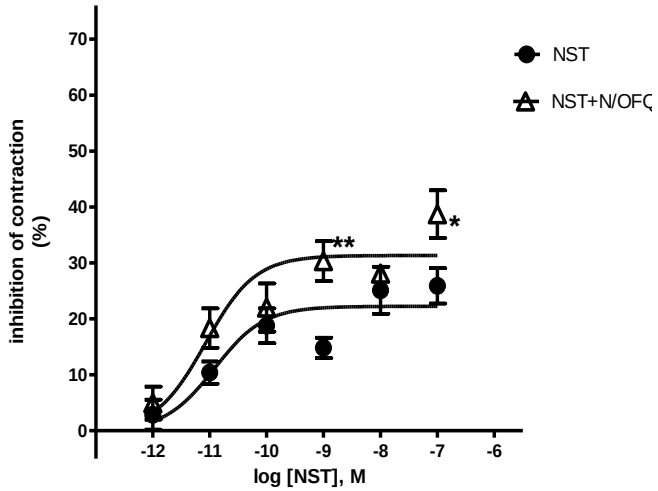
The KCl-evoked rhythmic contractions in the pregnant rat uterus were inhibited in a concentration-dependent manner by N/OFQ. In the presence of the opioid antagonist NX, the maximum contraction-inhibiting effect of N/OFQ was increased (**Figure 2A, Table 3**). NX alone caused a non-significant inhibition of the KCl-evoked contractions (n=8 for each set of experiments; data not shown).

The contraction-inhibitory effect of NST was investigated in three different agonist-induced contractions. The oxytocin-, $PGF_{2\alpha}$- or KCl-stimulated contractions did not decrease significantly through the experiment. Oxytocin was able to contract the uterine smooth muscle, but in the presence of oxytocin, NST alone or in the presence of N/OFQ (10^{-8} M), did not significantly reduce the contractions (data not shown). In the case of $PGF_{2\alpha}$-induced contractions, NST and N/OFQ displayed slight inhibitory effects (**Fig. 2B**). There was no significant difference between the $logEC_{50}$ values. NST alone decreased the KCl-induced contractions concentration-dependently. Co-administration of N/OFQ (10^{-8} M) with NST, however, significantly increased the maximum contraction-inhibitory effect of NST; $P<0.05$ (**Fig. 2C**). There was no significant difference between the $logEC_{50}$ values (**Table 4**).
The maximum inhibitory effect of NST was decreased by NX (10^{-8} M); $P<0.001$. There was no significant difference between the $logEC_{50}$ values (**Table 6**). NX also decreased the maximum contraction-inhibitory effect of the combination NST+N/OFQ; $P<0.05$ (**Fig. 2C**). There was no significant difference between the $logEC_{50}$ values (**Table 4**).

A.



B.



C.

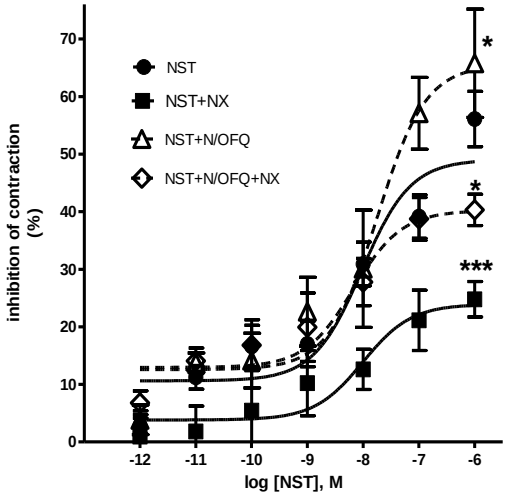


Fig. 2. Inhibitory effects of nociceptin (N/OFQ), nocistatin (NST) and naloxone (NX) on pregnant rat uterine contractions *in vitro*.

A) The contractions were elicited with 25 mM KCl in 22-day-pregnant rat uterine rings. The concentration-dependent inhibitory effect of N/OFQ (▲) was potentiated by the presence of 10^{-8} M NX (■). NX alone caused a non-significant inhibition of KCl-evoked contractions (data not shown); n=8. **B)** The contractions were elicited with 1 μM prostaglandin F2 alpha. The concentration-dependent inhibitory effect of NST (●) was increased by N/OFQ (Δ); n=6. **C)** The contractions were elicited with 25 mM KCl in 22-day-pregnant rat uterine rings. The concentration-dependent inhibitory effect of NST (●) was increased by N/OFQ (Δ); n=6. The concentration-dependent inhibitory effect of NST (●) was significantly attenuated by NX (■); n=10. When NST, N/OFQ and NX were present, the joint effect of the three drugs (◊) was decreased as compared with the dual effect of NST and N/OFQ (Δ); n=6.

Substance	**logEC$_{50}$** (± S.E.M.)		**E$_{max}$** (% ± S.E.M.)	
N/OFQ	-10.03 ± 0.40		27.55 ± 3.09	
N/OFQ+NX	-10.48 ± 0.30	ns	39.27 ± 3.18	*****

Table 3. logEC$_{50}$ and maximum contraction-inhibitory values of nociceptin (N/OFQ) alone and in the presence of naloxone (NX) on KCl-stimulated uterine contractions in the 22-day-pregnant rat *in vitro* (n=8). *****P<0.05, ns: nonsignificant; significances are expressed relative to N/OFQ.

Substance	**logEC$_{50}$** (± S.E.M.)		**E$_{max}$** (% ± S.E.M.)	
NST (on PGF$_{2\alpha}$-evoked contraction)	-10.92 ± 0.22		25.90 ± 3.16	
NST + N/OFQ	-11.07 ± 0.21	ns	38.71 ± 4.26	*****
NST (on KCl-evoked contraction)	-8.02 ± 0.18		56.10 ± 4.82	
NST + N/OFQ	-7.75 ± 0.27	ns	65.78 ± 9.42	*****
NST + N/OFQ + NX	-8.17 ± 0.27	**b**	40.33 ± 2.73	**a**

Table 4. logEC$_{50}$ and maximum contraction-inhibitory values of nocistatin (NST) alone and in the presence of nociceptin (N/OFQ) on prostaglandin F2$_{\alpha}$ (PGF$_{2\alpha}$) or KCl-stimulated contractions; and of NST with N/OFQ+NX on KCl-stimulated contractions in the 22-day-pregnant rat uterus *in vitro* (n=8).

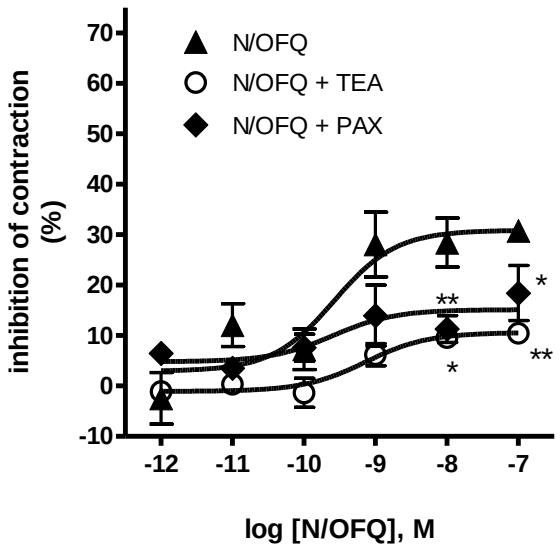
*****P<0.05, ns: nonsignificant; significances are expressed relative to NST alone. **a**: P<0.05, **b**: nonsignificant; significances are expressed relative to NST in the presence of N/OFQ.

4.1.4.2. Investigation of the role of BK_{Ca} channels in mediating the effects of N/OFQ and NST

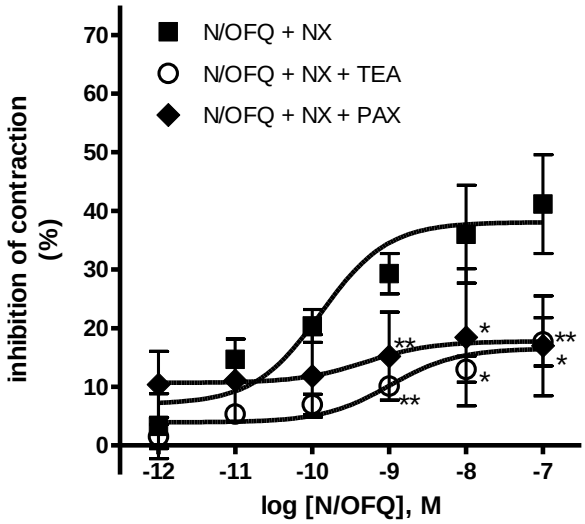
Because the central actions of N/OFQ and NX are mediated via the activation of the BK_{Ca} channels and hyperpolarization of the neurons, the effects of N/OFQ and NX on the uterine samples were also tested in the presence of the nonselective K^+ channel inhibitor TEA (10^{-3} M) and the BK_{Ca} channel-selective inhibitor PAX ($5x10^{-6}$ M) (**Fig. 3A, B**). In these experiments the rhythmic uterine contractions were evoked by oxytocin instead of KCl. TEA and PAX themselves did not change the pattern of oxytocin-induced contractions. However, the uterus-relaxing effect of N/OFQ on oxytocin-evoked rhythmic contractions was significantly attenuated by TEA and PAX. Similarly, when both N/OFQ and NX were present, their common contraction-inhibiting effect was significantly decreased by TEA and PAX (n=6 for each set of experiments).

The effects of NST on the spontaneous contractions of the term-pregnant rat uterus were also tested in the presence of the selective BK_{Ca} channel inhibitor PAX ($5x10^{-6}$ M), (**Fig. 3C**). In the presence of PAX, the maximum contraction-inhibitory effect of NST was decreased significantly, while there was no significant difference between the $logEC_{50}$ values (**Table 5**).

A.



B.



C.

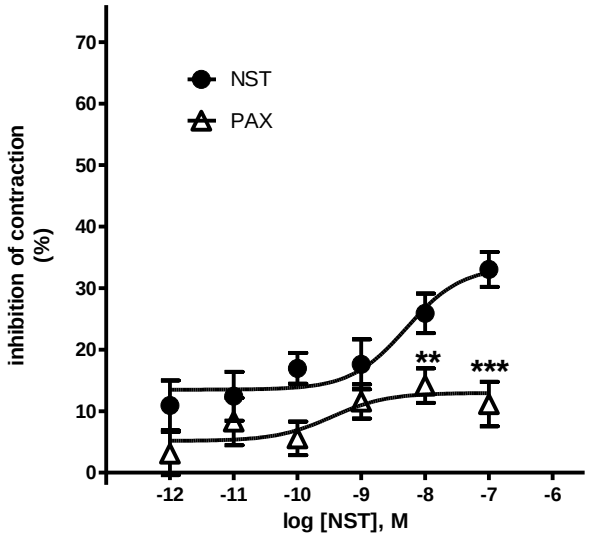


Fig. 3. Effects of potassium channel inhibitors on the pregnant contraction-inhibitory effects of nociceptin (N/OFQ) and nocistatin (NST) *in vitro*. A) The contractions were elicited with 10^{-8} M oxytocin in 22-day-pregnant uterine rings from the rat. The concentration-dependent relaxing effect of N/OFQ (▲) was decreased both by the nonselective K^+ channel inhibitor tetraethylammonium (TEA; 10^{-3} M; ○) and by the outward rectifying K^+ channel inhibitor paxilline (PAX; $5x10^{-6}$ M; ◆). No significant difference was found between the effects of the two K^+ channel blockers (n=6). **B)** The relaxing effect of N/OFQ was slightly increased by naloxone (NX; 10^{-8} M; ■). Their joint effect was decreased both by the nonselective K^+ channel inhibitor TEA (10^{-3} M; ○) and by the outward rectifying K^+ channel inhibitor PAX ($5x10^{-6}$ M; ◆). No significant difference was found between the effects of the two K^+ channel blockers (n=6). **C)** The spontaneous contractions were recorded in 22-day-pregnant uterine rings from the rat. The concentration-dependent inhibitory effect of NST (●) was decreased by PAX (Δ); n= 8.

Substance	**logEC$_{50}$** (± S.E.M.)		**E$_{max}$** (% ± S.E.M.)	
N/OFQ (oxytocin)	-9.58 ± 0.40		30.89 ± 3.65	
N/OFQ+TEA	-9.14 ± 0.34	ns	10.60 ± 1.73	**
N/OFQ+PAX	-9.60 ± 0.83	ns	15.14 ± 2.73	*
N/OFQ+NX	-9.89 ± 0.34		38.07 ± 3.95	
N/OFQ+NX+TEA	-8.99 ± 0.42	**c**	16.54 ± 2.12	**a**
N/OFQ+NX+PAX	-9.12 ± 0.90	**c**	17.75 ± 2.32	**b**
NST (spontaneous)	-8.29 ± 0.45		33.04 ± 2.83	
NST + PAX	-9.47 ± 0.93	**d**	11.15 ± 3.61	***

Table 5. $logEC_{50}$ and maximum inhibitory effects of nociceptin (N/OFQ) alone or combined with naloxone (NX) in the presence of tetraethylammonium (TEA; 10^{-3} M) or paxilline (PAX; $5x10^{-6}$ M) on oxytocin-evoked uterine contractions; and of nocistatin (NST) in the presence of PAX ($5x10^{-6}$ M) on spontaneous uterine contractions in the 22-day-pregnant rat *in vitro*. *P<0.05, **P<0.01, ns:nonsignificant; significances are expressed relative to N/OFQ alone. **a**: P<0.05, **b**: P<0.01, **c**: nonsignificant; significances are expressed relative to N/OFQ+NX. ***P<0.001, **d**: nonsignificant; significances are expressed relative to NST.

4.1.4.3. Investigation of the contraction-inhibitory effects of NST and NX in a hypocalcemic environment

In the hypocalcemic environment, the concentration—response curves of NST alone and of NST in combination with NX were both shifted to the left as compared with the curves in standard de Jongh solution. The $logEC_{50}$ values of NST alone and of NST with NX were significantly lower in the hypocalcemic environment than in standard de Jongh solution (P<0.05). At the same time, NX did not decrease the inhibitory effect of NST in the hypocalcemic environment as it did in the standard buffer. The maximum contraction-inhibitory effect of NST alone did not differ from that of NST in combination with NX in the hypocalcemic buffer (**Fig. 4**, **Table 6**).

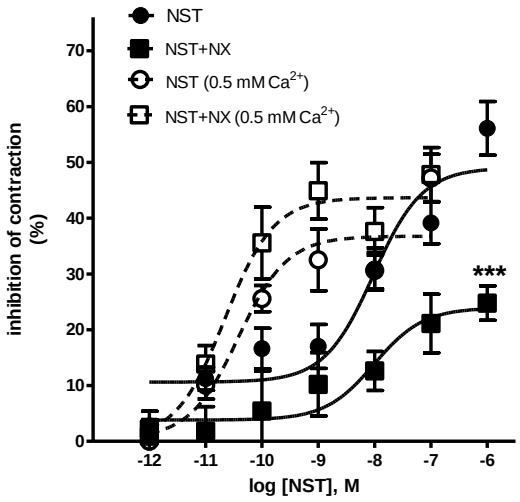


Fig. 4. Effects of nocistatin (NST) and naloxone (NX) on pregnant uterine contractions in a hypocalcemic environment *in vitro*.

The contractions were elicited with 25 mM KCl in 22-day-pregnant rat uterine rings. The concentration-dependent inhibitory effect of NST (○) was not altered by NX (□) in the hypocalcemic buffer, whereas it was inhibited in the standard Ca^{2+}-containing buffer (● NST; ■ NX); n= 6.

Substance	**logEC$_{50}$** (± S.E.M.)		**E$_{max}$** (% ± S.E.M.)	
NST (1 mM Ca^{2+})	-8.02± 0.18		56.10 ± 4.82	
NST + NX (1 mM Ca^{2+})	-8.01± 0.47	ns	24.78 ± 3.08	**a**
NST (0.5 mM Ca^{2+})	-10.42± 0.23	**b**	47.21 ± 4.27	
NST + NX (0.5 mM Ca^{2+})	-10.66± 0.24	ns; **b**	47.82 ± 4.85	ns

Table 6. logEC$_{50}$ and maximum contraction-inhibitory effects of nocistatin (NST) alone and in the presence of naloxone (NX) on KCl-stimulated uterine contractions in the 22-day-pregnant rat *in vitro*, either in standard Ca^{2+}-containing or in hypocalcemic de Jongh solutions (n=6).
a: P<0.001, ns: nonsignificant. The significances of the joint effect of NST + NX are expressed relative to NST alone in the same Ca^{2+}-containing de Jongh solution. **b**: P<0.05; significances are expressed relative to the same substance in a different Ca^{2+}-containing de Jongh solution.

4.1.4.4. Investigation of the role of CGRP in mediating the effects of NST

Since the exact site of action of NST is still unclear, we tested whether it might act by modulating neuropeptide release from capsaicin-sensitive sensory nerve endings in the pregnant rat uterus. Neuropeptide depletion from the capsaicin-sensitive primary afferents was induced with capsaicin (**Fig. 5**). The maximum contraction-inhibitory effect of NST was decreased significantly (P<0.01) after preincubation with capsaicin (1 μM; **Table 7**). The solvent of capsaicin (control) did not change the effect of NST (P>0.05).

When the neuropeptide depletion was followed by the addition of CGRP (0.1 μM), the maximum contraction-inhibitory effect of NST was significantly higher than after incubation with capsaicin (P<0.01). The addition of CGRP restored the inhibitory effect of NST as compared with the control (P>0.05).

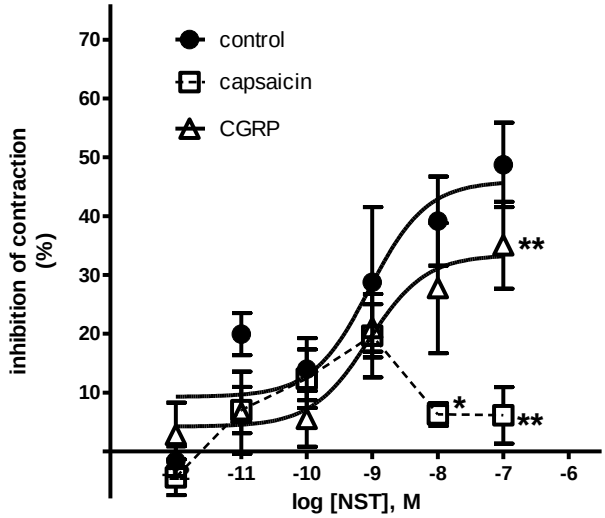


Fig. 5. The role of calcitonin gene-related peptide (CGRP) on the contraction-inhibitory effect of nocistatin (NST) *in vitro.*

The contractions were elicited with 25 mM KCl in 22-day-pregnant rat uterine rings. The concentration-dependent inhibitory effect of NST (●; control) was reduced significantly after preincubation with capsaicin (□; 1 μM). Addition of CGRP (0.1 μM) after preincubation with capsaicin caused a significant elevation of the concentration—response curve of NST (Δ); n= 6.

Substance	**logEC_{50}** (± S.E.M.)	**Max. inhibition at 10^{-7} M nocistatin** (% ± S.E.M.)	
NST control	-9.04 ± 0.43	48.72 ± 7.18	
NST preincubated with capsaicin	not converged	6.14 ± 4.79	**a**
NST preincubated with capsaicin and CGRP	-9.06 ± 0.46	35.05 ± 7.38	**a**

Table 7. logEC_{50} and maximum contraction-inhibitory effects of nocistatin (NST) after preincubation with capsaicin (1 μM), with the solvent of capsaicin (control) and with capsaicin (1 μM) and CGRP (0.1 μM) (n=6). **a**: P<0.01. Significances after preincubation with capsaicin are expressed relative to NST alone, and significances after preincubation with capsaicin and CGRP are expressed relative to preincubation with capsaicin alone.

4.1.5. Measurement of *PNOC* mRNA in the rat uterus

The myometrial *PNOC* mRNA levels increased significantly as term was approached. The PCR study showed that the levels of *PNOC* mRNA/*β-actin* mRNA and *PNOC* mRNA/*GAPDH* mRNA were lowest on pregnancy day 18. The relative expression of *PNOC* mRNA on day 20 was not different from that on day 18, but it was increased significantly by day 22, the day of delivery; P<0.001 (**Fig. 6**, the endogenous control was *β-actin;* we observed the same results with *GAPDH*, data not shown).

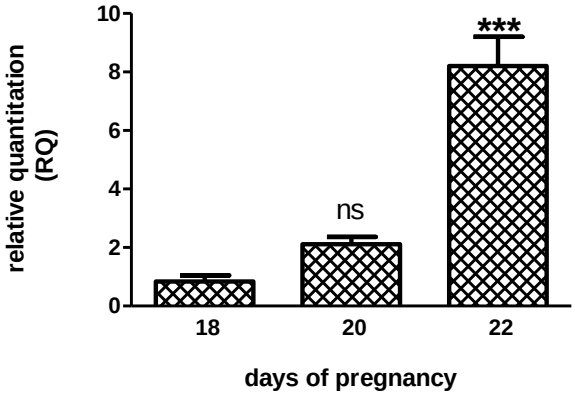


Fig. 6. Levels of expression of *PNOC* mRNA in the rat uterus on days 18, 20 and 22 of pregnancy

As term was approached, the *PNOC* mRNA level increased; there was a significant increase in the level of *PNOC* mRNA in uterus samples obtained from rats on day 22 (i.e. at term) as compared with that on day 20 of pregnancy (n=3); ***P<0.001, ns: non-significant. Significances are expressed relative to the previous column. The endogenous control is *β-actin*.

4.1.6. Measurement of uterine cAMP accumulation

In order to investigate the mechanism by which N/OFQ inhibits uterine contractions, the effect of N/OFQ on uterine cAMP accumulation was also measured (**Fig. 7**). The N/OFQ (10^{-8} M) and NX (10^{-8} M)-stimulated uterine cAMP accumulations were detected in the presence of the non-specific phosphodiesterase inhibitor IBMX (10^{-3} M) and the adenylyl cyclase activator forskolin (10^{-5} M). N/OFQ alone did not evoke a significant increase ($P>0.05$) in the uterine cAMP accumulation (14.0 ± 0.6 pmol/mg tissue) as compared with the non-treated control samples (13.18 ± 0.2 pmol/mg tissue). However, its combination with NX caused a significant elevation ($P<0.05$) in the uterine cAMP level (15.0 ± 0.7 pmol/mg tissue). Moreover, if the uterine tissue samples were preincubated with the G_i protein inhibitor PTX (500 ng/ml), N/OFQ with NX elevated the uterine cAMP level far higher ($P<0.01$) than that without preincubation (36.5 ± 0.5 pmol/mg tissue), which points to the involvement of G_s-proteins in the intracellular signalling pathways of N/OFQ and NX in the pregnant rat uterus (n=6 for each set of experiments).

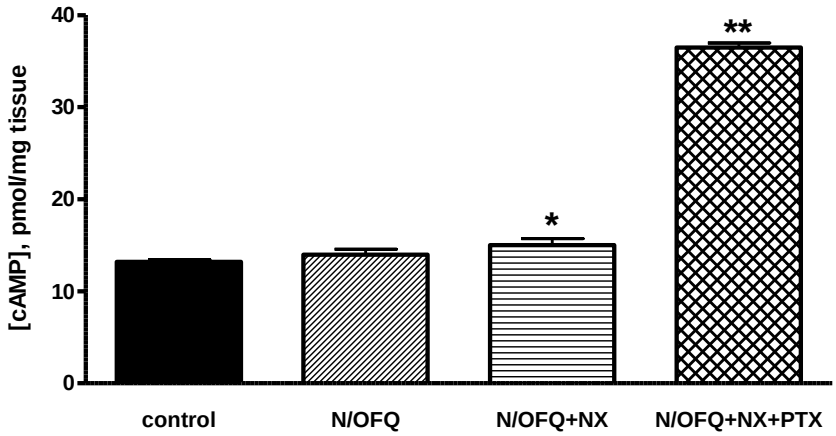


Fig. 7. Effects of nociceptin (N/OFQ) on the intracellular cAMP levels in the pregnant rat myometrium. N/OFQ itself did not increase the cAMP level as compared with the control (black column). The presence of naloxone (N/OFQ+NX) increased the N/OFQ-induced intracellular cAMP level, while the addition of pertussis toxin (N/OFQ+NX+PTX) resulted in a robust elevation in cAMP level. n=6; *P<0.05, **P<0.01.

We also investigated whether cAMP accumulation plays a role in the contraction-inhibitory effect of NST (**Fig. 8**). NST evoked a significant increase ($P<0.001$) in the uterine cAMP level (15.12 ± 0.40 pmol/mg tissue) as compared with the basic activity (12.82 ± 0.17 pmol/mg tissue). Co-administration of N/OFQ with NST caused a further elevation in the cAMP level (17.48 ± 0.29 pmol/mg tissue; $P<0.01$). However, when NX was co-administered with NST, a significant decrease was detected in the cAMP level (13.35 ± 0.52 pmol/mg tissue) as compared with the effect of NST alone ($P<0.05$).

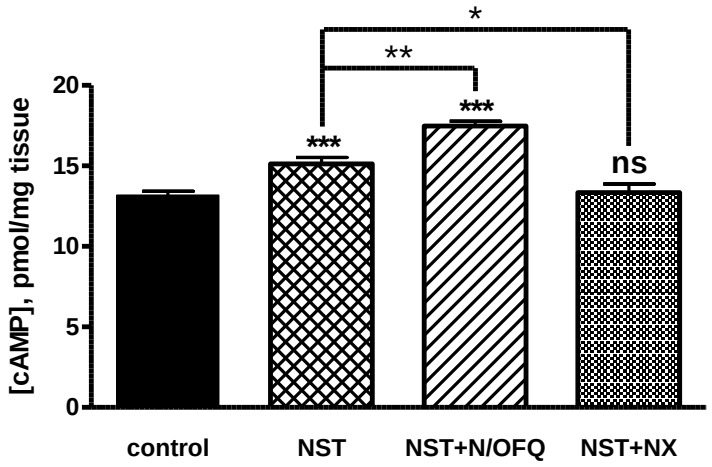


Fig. 8. Effects of nocistatin (NST) on intracellular cAMP levels in the pregnant rat uterus.

NST caused a significant elevation in cAMP level as compared with the control (black column); ***$P<0.001$. The presence of N/OFQ increased (**$P<0.01$), while NX decreased (*$P<0.05$) the NST-induced intracellular cAMP accumulation; n=6.

4.2. Investigations on pregnant human myometrium obtained from full-term pregnancy and from preterm birth

4.2.1. *In vitro* contractility studies in the full-term pregnant human myometrium

N/OFQ alone decreased the uterine contractility concentration-dependently. NST (10^{-8} M) increased the maximum contraction-inhibitory effect of N/OFQ significantly ($P<0.001$; **Fig. 9A**, **Table 8**). There was no significant difference between the $logEC_{50}$ values (data not shown). NST alone exerted a contraction-inhibitory effect. However, co-administration of N/OFQ (10^{-8} M) with NST did not alter the inhibitory effect of NST ($P>0.05$; **Fig. 9B**). There was no significant difference between the $logEC_{50}$ values (data not shown).

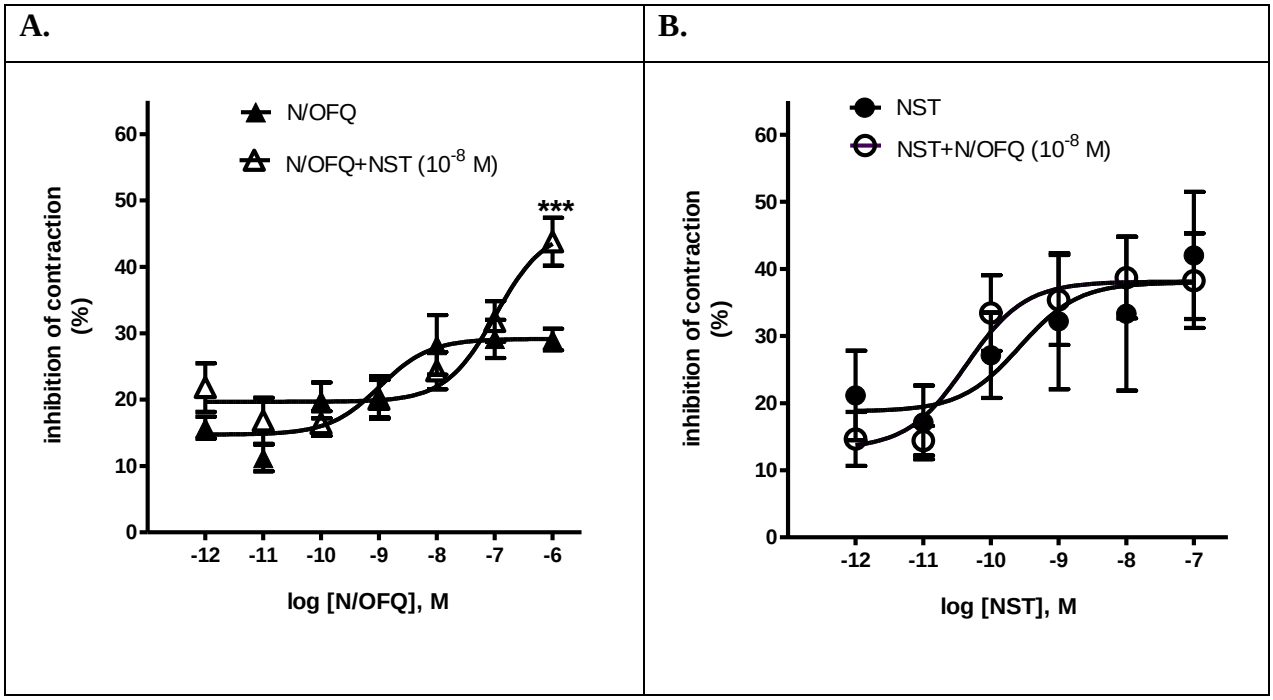


Fig. 9. Uterus-relaxant effects of nociceptin (N/OFQ) and nocistatin (NST) on the term-pregnant human myometrium *in vitro*. The contractions were elicited with 10^{-8} M oxytocin in uterine rings from full-term human pregnancies. **A)** The concentration-dependent inhibitory effect of N/OFQ (▲) was significantly increased in the presence of NST (10^{-8} M; Δ); n=6. **B)** The concentration-dependent inhibitory effect of NST (●) was not altered by N/OFQ (10^{-8} M; ○); n=4.

4.2.2. *In vitro* contractility studies in the pregnant human myometrium from preterm births

N/OFQ alone decreased the uterine contractility concentration-dependently. NST (10^{-8} M) increased the maximum contraction-inhibitory effect of N/OFQ significantly (P<0.001; **Fig. 10A, Table 8**). There was no significant difference between the $logEC_{50}$ values (data not shown). NST alone demonstrated a contraction-inhibitory effect, which was not altered by N/OFQ (P>0.05; **Fig. 10B**). There was no significant difference between the $logEC_{50}$ values (data not shown).

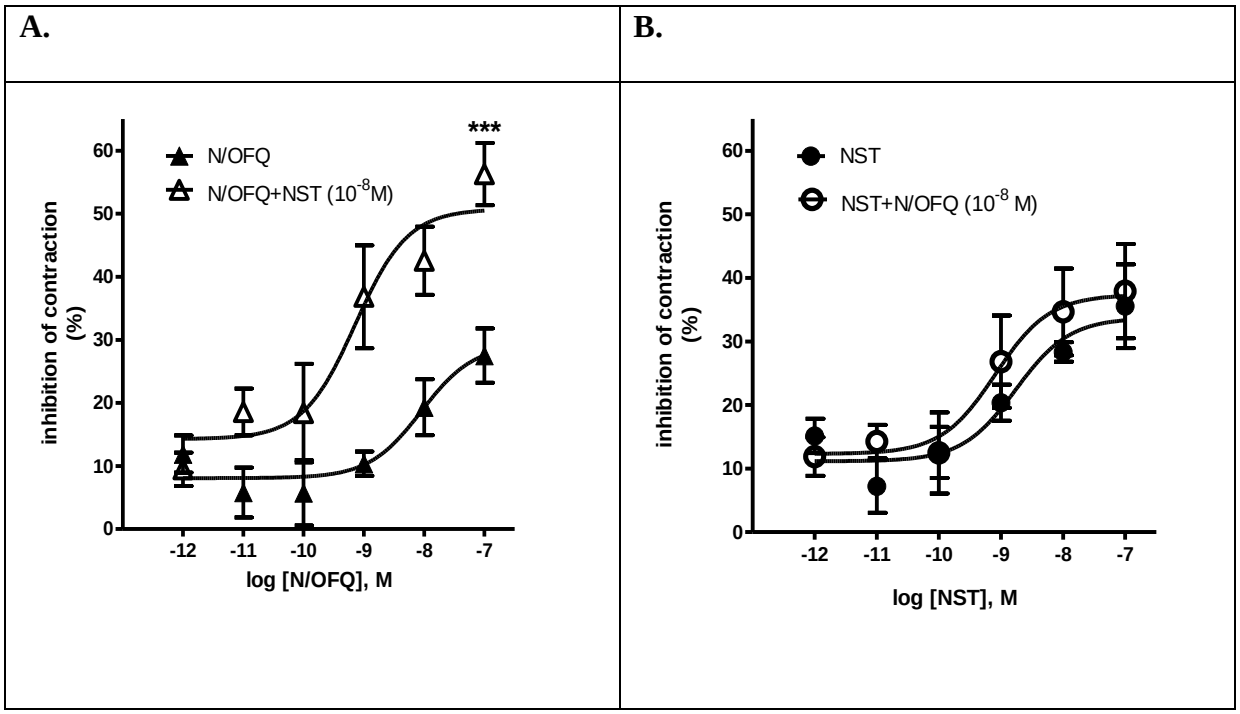


Fig. 10. Uterus-relaxant effects of nociceptin (N/OFQ) and nocistatin (NST) on the preterm-pregnant human myometrium *in vitro*. The contractions were elicited with 10^{-8} M oxytocin in uterine rings from preterm human pregnancies. **A)** The concentration-dependent inhibitory effect of N/OFQ (▲) was significantly increased in the presence of NST (10^{-8} M; Δ); n=4. **B)** Co-administration of N/OFQ (10^{-8} M) with NST (○), however, did not significantly alter the inhibitory effect of NST (●); n=5.

	E_{max} (% ± S.E.M.)			E_{max} (% ± S.E.M.)	
Term-pregnant uterus			Preterm-pregnant uterus		
N/OFQ	29.07 ± 1.61		N/OFQ	27.51 ± 4.31	
N/OFQ + NST	43.79 ± 3.61	***	N/OFQ + NST	56.29 ± 4.94	***
NST	42.01 ± 9.48		NST	35.55 ± 6.58	
NST + N/OFQ	38.24 ± 7.05	ns	NST + N/OFQ	37.92 ± 7.40	ns

Table 8. Maximum contraction-inhibitory values of nociceptin (N/OFQ) alone and in the presence of nocistatin (NST); and of NST alone and in the presence of N/OFQ on oxytocin-stimulated uterine contractions on the term- and preterm-pregnant human myometrium *in vitro*. ***P<0.001; significances are expressed relative to N/OFQ. ns: non-significant; significances are expressed relative to NST.

4.2.3. Measurement of *PNOC* mRNA in the human uterus

The myometrial *PNOC* mRNA levels were significantly higher in preterm uterine samples (RQ=35.40 ± 3.31) as compared with samples from full-term pregnancy (RQ=0.81 ± 0.12); P<0.001 (**Fig. 11**).

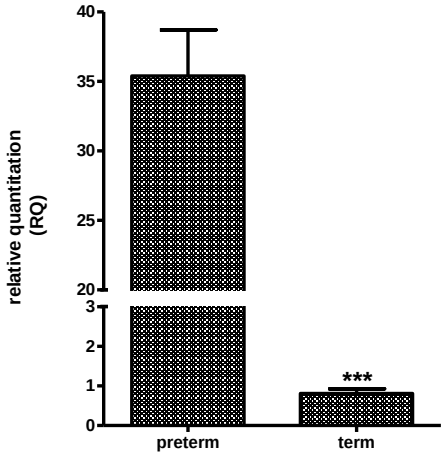


Fig. 11. Levels of expression of *PNOC* mRNA in human uterus samples obtained from preterm birth and full-term birth. By term pregnancy, the *PNOC* mRNA level was decreased; there was a significant decline in the level of *PNOC* in full-term uterine samples as compared with preterm samples; ***P<0.001.

5. Discussion

PNOC mRNA is expressed predominantly in the central nervous system (the brain and spinal cord). A previous study also confirmed its presence in human peripheral blood mononuclear cells (Williams *et al.*, 2008), and it has been detected in the rat ovary (Mollereau *et al.*, 1996 and Leo *et al.*, 2001). In the human brain, Foradori et al. (Foradori *et al.*, 2007) found that gonadotropin releasing hormone (GNRH) immunoreactive nerve cells co-localize N/OFQ in a high percentage. These findings strongly indicate that the actions of GNRH are modulated in part by N/OFQ, this mechanism controlling the reproductive functions by regulating release of the gonadotropic hormones FSH and LH from the pituitary. However, the expressions of *PNOC* mRNA as well as the presence and effects of N/OFQ and NST have not been investigated to date in the female rat and human uterus.

5.1. Myometrial expression of PNOC mRNA, NOP, N/OFQ and NST

We found that the common precursor for N/OFQ and NST, the *PNOC* mRNA, is expressed locally in the pregnant rat and human uterus, indicating that N/OFQ and NST are synthesized locally. The expression of *PNOC* mRNA was high on the last day of pregnancy in the rat as compared with earlier days. The RIA results confirmed the presence of both N/OFQ and NST in the nonpregnant and the pregnant rat uterus. Furthermore, we found that NST in the pregnant rat uterus at term is about 10 times more abundant than N/OFQ, so it seems that *PNOC* mRNA is translated mainly to NST, rather than N/OFQ. As regards the higher contractility during delivery, the elevation of N/OFQ and NST levels in the myometrium at term appears rather contradictory, since they have uterus relaxing effect. Nevertheless, they have regulatory role in pain signalling, which may explain their functional importance during labour. In the human samples PNOC shows an elevated level in preterm pregnancies, which is in correlation with the uterine quiescence during pregnancy, although it is contrary to the results obtained from the rat. Much more NST and N/OFQ can be translated from PNOC in preterm pregnancy than in the term pregnancy. The PNOC level drops by the end of pregnancy, when uterine contractility becomes stronger.

We also detected specific binding sites for NOP both in the nonpregnant and term-pregnant rat uterus, their concentration being similar to that in most brain regions (Clarke *et al.*, 2003). This may suggest that pro-nociceptive peptides are likely to be involved in local mechanisms regulating the uterine functions at term.

5.2. In vitro contractility studies

The idea that endogenous pro-nociceptive substances might have a modulating effect on the uterine smooth muscle contractility stems from the ancient experience that labour contractions are extremely painful. Thus, labour pain and powerful contractions are usually related.

As regards its effects on the uterus, N/OFQ was found to inhibit both KCl- and oxytocin-evoked rhythmic contractions. In order to exclude the possible involvement of classical opioid receptors in the mediation of this effect, NX was co-administered with N/OFQ onto the isolated uterine tissue samples. Our results revealed that NX significantly increased the uterus-relaxing effect of N/OFQ, suggesting that NX has a relaxing effect on its own. The effect of NX alone on the uterus appeared to be nonsignificant. The potentiating effect of NX may seem unexpected, but a publication reported an elevated uterine tone in humans after intrathecal administration of opioids during labour (Abrão *et al.*, 2009), which suggests that endogenous opioids might have a contraction-enhancing effect on the uterus that can be attenuated by NX.

Although NST possesses a contraction-inhibitory effect on KCl- and $PGF_{2\alpha}$-evoked contractions, its action was weak against $PGF_{2\alpha}$. N/OFQ was able to potentiate the inhibitory effect of NST in both cases. NST does not counteract the effects of N/OFQ on the myometrium contractility, as was presumed in previous studies relating to their actions in the central nervous system. However, NST was ineffective on oxytocin-induced contractions. This phenomenon will be discussed later (see Section 5.6.) with regard to a detailed mechanism of the action of NST on the pregnant rat myometrium.

NX inhibits the contraction-inhibitory effect of NST against KCl-induced contractions. NST does not bind to any of the classical opioid receptors, or to the NOP receptor (Johnson and Connor, 2007 and Fantin *et al.*, 2007), thus this inhibition is not likely to be mediated by opioid receptors. It was demonstrated that NX induces an increase in inward Ca^{2+} currents (Kai *et al.*, 2002). These findings led us to investigate whether the inhibitory effect of NX on NST-induced uterus relaxation is mediated by the opening of inward rectifying Ca^{2+} channels. To test our hypothesis, we used a hypocalcemic environment, where NX did not inhibit the effect of NST. We presume that, in a low extracellular Ca^{2+} concentration, NX is probably unable to promote a Ca^{2+} influx, and hence it can not overcome the relaxation induced by NST. The leftward shift in the concentration—response curves are likely to be due to the weaker tissue contractility, caused by the lower Ca^{2+} content. When N/OFQ was present, NX was able to decrease the common contraction-inhibitory effect of NST and N/OFQ. However,

although NX increases the effect of N/OFQ, the effect seen here is probably the consequence of the more pronounced inhibitory effect of NST as compared with that of N/OFQ.

5.3. Investigation of G protein-activating potency

After the description of the contraction-inhibitory effect of N/OFQ and NST in the *in vitro* contractility studies, our further aim was to investigate some of the potential signalling pathways.

The high homology between the NOP receptor and the three opioid receptor subtypes raised the question of whether the NOP receptor is a G_i coupled receptor that regulates adenylyl cyclase activity, K^+ channels and voltage-gated Ca^{2+} channels. The cellular actions of N/OFQ were found to be similar to those of opioids, i.e. inhibition of the formation of cAMP, closure of voltage-sensitive Ca^{2+} channels, and enhancement of outward K^+ conductance (Hawes *et al.*, 2000). Hence, we also tested the G protein-activating effect of N/OFQ on the term-pregnant rat uterus, and found a significant elevation of [^{35}S]GTPγS binding through the NOP receptors. Thus, G proteins at least in part, mediate the actions of N/OFQ on the uterus. Furthermore, the presence of NX decreased the maximum [^{35}S]GTPγS binding and at the same time increased the uterus-relaxing effect of N/OFQ. A possible explanation for this phenomenon might be that NX interferes with the G_i protein-activating potency of N/OFQ. In the presence of NX and PTX, which uncouples the receptor from the G_i protein and prevents the inhibition of adenylyl cyclase activity, we detected a dramatic fall in N/OFQ-induced G protein production. This inhibition of the G_i proteins resulted in activation below the basal level. The phenomenon that individual receptors are able to activate multiple pathways by switching between different G proteins is well established (Lefkowitz *et al.*, 2002; Knollman *et al.*, 2008). The coupling of beta-adrenergic receptors to G_s proteins leads to the activation of adenylate cyclase and the consequent phosphorylation of protein kinase A (PRKA), which phosphorylates the receptor and diminishes its coupling to G_s, but increases the coupling to G_i. We presume that NX interferes with the intracellular pathways activated by N/OFQ and promotes its coupling to G_s. This mechanism results in an elevation of the myometrial cAMP level and uterus relaxation. N/OFQ can effectively activate protein kinase C via NOP receptor, through a pertussis toxin (PTX)-sensitive G-protein activation ($G_{i/0}$). Besides, it has been shown, that phospholipase C (PLC) inhibitors block this pathway, which means, that Ca^{2+} concentration is increased, and thus phospholipase C activation are involved in the signalling mechanism (Lou *et al.*, 1997).

5.4. Measurement of cAMP accumulation

In order to investigate further the mechanism by which N/OFQ and NST inhibit uterine contractions, the effects of N/OFQ and NST on uterine cAMP accumulation were also measured. Co-administration of NX with N/OFQ increased the uterine cAMP level. To test the participation of G_s proteins in the actions of N/OFQ, the changes in cAMP level in the uterus were assessed in the presence of PTX. In this experiment, the cAMP level rose markedly, which suggests that NOP receptors are probably coupled to both G_i and G_s proteins in the late-pregnant rat uterus, and G_s stimulation is responsible for the elevated cAMP level. We also presume that N/OFQ and NX compete for intracellular G_i protein activation. We detected elevation of cAMP levels in the presence of NST. In the presence of N/OFQ with NST, a further cAMP level elevation was found, which can be explained by the mutual cAMP-accumulating effects of N/OFQ and NST-induced CGRP liberation (Klukovits *et al.,* 2010 and Dong *et al.,* 2005). In correlation with the *in vitro* contractility studies, NX decreased the cAMP levels elevated by NST, which suggests that NX interferes with NST at the level of G-protein activation, too.

5.5. Investigation of the role of K^+ channels in mediating the effects of N/OFQ and NST

However, the changes in local cAMP levels do not seem to be the only intracellular pathway regulated by N/OFQ and NST. N/OFQ has been reported to enhance an outward K^+ conductance, which reduces neuronal excitability and transmitter release in the brain (Hawes *et al.*, 2000). In the uterus, BK_{Ca} channels are abundant and play an important role in limiting depolarization, thereby relaxing the uterine smooth muscle. Moreover, these BK_{Ca} channels are activated by a cAMP-dependent phosphorylation pathway (Chanrachakul *et al.*, 2004). We set out to investigate the role of K^+ currents in the intracellular signalling of N/OFQ and NST. We found that blockade of the K^+ channels with TEA or PAX diminishes the uterus-relaxing effect of N/OFQ, applied either alone or in combination with NX, which suggests that this effect of N/OFQ on the pregnant rat uterus is mediated by the activation of K^+ channels. There was no difference between the N/OFQ-inhibiting effects of the non-selective K^+ channel blocker TEA and the BK_{Ca}-selective blocker PAX. This result suggests that BK_{Ca} channels may have a crucial role in the uterus-relaxing effect of N/OFQ. In the presence of TEA or PAX, N/OFQ alone or in combination with NX still produced a limited uterus-relaxing effect, which is suspected to be mediated by the elevated cAMP level. PAX inhibited the contraction-inhibitory effect of NST, as evidence that the Ca^{2+}-dependent K^+ channels play a role in the intracellular signalling of NST as well.

5.6. Role of sensory neuropeptide CGRP

It is known, that inflammatory mediators play a role in the initiation of labour, yet some of them (e.g. CGRP) exhibit utero-relaxant activity among their various effects. Opioid-like nociceptive peptides have been reported to release neurotransmitters such as CGRP or substance P (SP) from capsaicin-sensitive primary sensory neurons (Peiser *et al.*, 2000 and Helyes *et al.*, 1997). These neuropeptides are synthesized in the dorsal root ganglia (DRG) cells, stored in vesicles and released by exocytosis (Lundberg *et al.*, 1996) in response to electrical (Markowitz *et al.*, 1987 and Buzzi *et al.*, 1991) or chemical stimulation (Kilo *et al.*, 1997). CGRP has been reported to inhibit smooth muscle contractility in a variety of tissues, including the pregnant rat uterus (Pennefather *et al.*, 1990). Other studies have reported that the binding of ^{125}I-CGRP to rat uterine membranes was increased during pregnancy and decreased during parturition (Yallampalli *et al.*, 1999).

The potential involvement of CGRP in the actions of NST on the pregnant rat uterus was therefore also tested. While capsaicin causes the depletion of CGRP from sensory nerve terminals (Holzer, 1991), other studies have furnished evidence of a CGRP reload into the sensory nerve terminals after depletion by capsaicin (Sams-Nielsen *et al.*, 2001). We investigated the effect of NST either on capsaicin-induced CGRP-depleted uterus samples or on CGRP-reloaded uterus samples. Capsaicin blocked the contraction-inhibitory effect of NST, which was restored after the tissue samples were incubated with CGRP. Consequently, we assume that CGRP is an important factor in the contraction-inhibitory effect of NST. To support the hypothesis of the cross-talk between NST and CGRP, a special population of opioid receptors in DRG neurons was reported, where low doses of opioids can provoke hyperalgesia, due to the activation of excitatory opioid receptors on the afferent nerve terminals (Crain and Shen, 2000). This phenomenon involves an increased release of excitatory neuropeptides, including CGRP and SP, in the spinal cord (Xu *et al.*, 2003, Wiesenfeld-Hallin *et al.*, 1991 and Crain and Shen, 1990]. We assume that NST, similarly to opioid peptides and N/OFQ, may promote the release of neuropeptides from sensory nerves (Moran *et al.*, 2000). This mechanism may explain the ineffectiveness of NST against oxytocin-induced contraction; it was reported that the CGRP-agonist adrenomedullin also failed to block oxytocin-evoked contractions, whereas it was effective against spontaneous and bradykinin-induced contractions (Yanagita *et al.*, 2000).

In the human tissues NST administration must precede the administration of N/OFQ with the aim of enhancing the common uterus-relaxant effect. We assume that the CGRP-liberating effect of NST results in a weaker relaxation as compared with the potassium channel opening

effect of N/OFQ; repeated and increasing administrations of N/OFQ following NST can therefore further increase the uterus relaxation through the potassium channels. On the other hand, if N/OFQ is administered first, the CGRP liberating effect of NST cannot exceed the relaxation caused by a single dose of N/OFQ through potassium channel opening, therefore the relaxing effect of NST cannot be potentiated by N/OFQ significantly. The more prominent potentiating effect of NST in N/OFQ-stimulated uterus relaxation in preterm birth as compared with term pregnancy correlates with the finding that PNOC is more abundant in preterm birth.

As NOP is expressed in a high proportion of substance P (SP)/CGRP-positive neurons, and as a major subpopulation of N/OFQ neurons is located in juxtaposition to SP/CGRP-positive neurons, it is proposed that N/OFQ released locally in the dorsal root ganglia may (in a paracrine manner) modulate SP/CGRP-containing neurons expressing NOP. Thus, N/OFQ modulates both central and peripheral SP- or CGRP-mediated neurotransmission (Mika *et al.*, 2003).

6. Conclusions

These results provide evidence that both N/OFQ and NST generated locally in the uterus have contraction-inhibitory effect in the pregnant rat and human uterus, and when they are administered together, they can potentiate each others effect, this mechanism being mediated mainly by BK_{Ca} channels and consequent hyperpolarization. NST additionally acts by release of the sensory neuropeptide CGRP. NX shows an additive relaxant effect with those of N/OFQ, on the other hand it overcomes the relaxation caused by NST by activating inward rectifying Ca^{2+} channels and by decreasing the cAMP-accumulating effect of NST. We have provided evidence that the NOP receptors are coupled to multiple G proteins. We assume that, N/OFQ or NOP-related (preferably non-peptide) agonists as well as NST derivatives might be considered as prospective candidates for tocolytic therapy. The findings of this *in vitro* study need to be evaluated under *in vivo* conditions, and further experiments on human tissue are necessary in order to allow conclusions on the relevance of the present findings as concerns human disease.

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