Final report

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Title: **Elucidation of the molecular mechanisms underlying the actions of opioid-like peptides nocistatin and nociceptin / orphanin FQ in the relaxation of the late-pregnant rat and human uterus**

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1. Introduction

Nocistatin (NST) and nociceptin / orphanin FQ (N/OFQ) are two neuropeptides that are derived from the same precursor prepronociceptin (PNOC), and have physiological roles in nociception [Okuda-Ashitaka et al., 1998; Martin et al., 1998; Meunier et al., 1995; Reinscheid et al., 1995]. Regarding its effect on the central nervous system, N/OFQ is considered to act as an important neuroendocrine regulator of female reproductive functions [Foradori et al., 2007]. The direct, peripheral actions of N/OFQ and NST have been sparsely investigated yet.

In this project we intended to focus on the possible involvement of NST, another peptide derived from the same precursor protein as N/OFQ, in the regulation of uterine contractility in the rat. We also aimed to find evidence for the participation of N/OFQ and NST in the control of human myometrial activity at term, with a theoretical perspective to provide suggestions for future tocolytic therapy.

2. Experiments on pregnant rats

2.1. Animals

The animals used in the experiments 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.

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

Rat uterine prepronociceptin (*PNOC*) mRNA expressions were determined on selected days of late pregnancy (days 18, 20 and 22) by RT-PCR. The myometrial *PNOC* mRNA levels increased significantly as pregnancy progressed: the levels of *PNOC* mRNA/*β-actin* mRNA were lowest on day 18 of pregnancy. The relative expression of *PNOC* mRNA on day 20 was not different from that on day 18, but it was increased by day 22, the day of delivery. With this experiment, it was confirmed for the first time, that *PNOC* mRNA is expressed in the pregnant rat uterus.

2.3. Radioimmunoassay (RIA) for NST in the rat uterus and plasma

RIA experiments were not planned in the project protocol. However, later we presumed it was necessary, to be able justify the translation of *PNOC* mRNA to NST protein in the rat uterus as well (similarly as we did before in case of N/OFQ, [Klukovits et al. 2010]). The extraction of NST from uterus tissue was carried out by a validated method. The RIA experiments revealed that the myometrial NST levels were relatively low on pregnancy days 18 and 20, then elevated significantly by day 22, the day of delivery (**Table 1**). The plasma levels of NST did not change with the progression of pregnancy. Furthermore, we found that NST in pregnant rat uterus at term is about 10 times more abundant than N/OFQ [Klukovits et al. 2010], so it seems that *PNOC* mRNA is translated mainly to NST, rather than to N/OFQ.

Table 1. Tissue and plasma NST levels in 18, 20 and 22 day pregnant rats (n=6).

Tissue	pg/100 mg uterine tissue ± S.E.M		pg/ml plasma ± S.E.M	
18 day pregnant	17.15±3.03	ns	11.52±1.62	ns
20 day pregnant	13.95±1.82	ns	12.19±1.57	ns
22 day pregnant	42.11±6.27	*	17.59±2.12	ns

*p<0.05, ns: nonsignificant. Significances are expressed relative to the value of the previous tested day.

As regards the higher spontaneous contractility during delivery, the elevation of NST levels in the myometrium at term appears rather contradictory, especially in the light of our results, assuming that NST has uterus relaxing effect. Nevertheless, it was also reported that NST inhibit hyperalgesia and allodynia, thus it has a regulatory role in pain signalling, which may

explain its functional importance during labour [Li et al. 2012; Minami et al. 1994a; Minami et al. 1994b; Taiwo et al. 1988].

2.4. In vitro contractility studies

These experiments were the most numerous during the run of the project. They were performed by Anna Klukovits, Principal Investigator and Beáta Deák, a PhD student mentioned in the project proposal.

In the isolated uterine rings from day-22 pregnant rats, rhythmic contractions were elicited with KCl, oxytocin (oxy) or with $PGF_{2\alpha}$ (**Figure 1**). NST acted as an inhibitor of the uterine contractions, evoked by the above contracting agents. The most potent inhibitory effect of NST was found against KCl, therefore this agent was used in the further studies to investigate the possible mechanism of action of NST (**Table 2**).

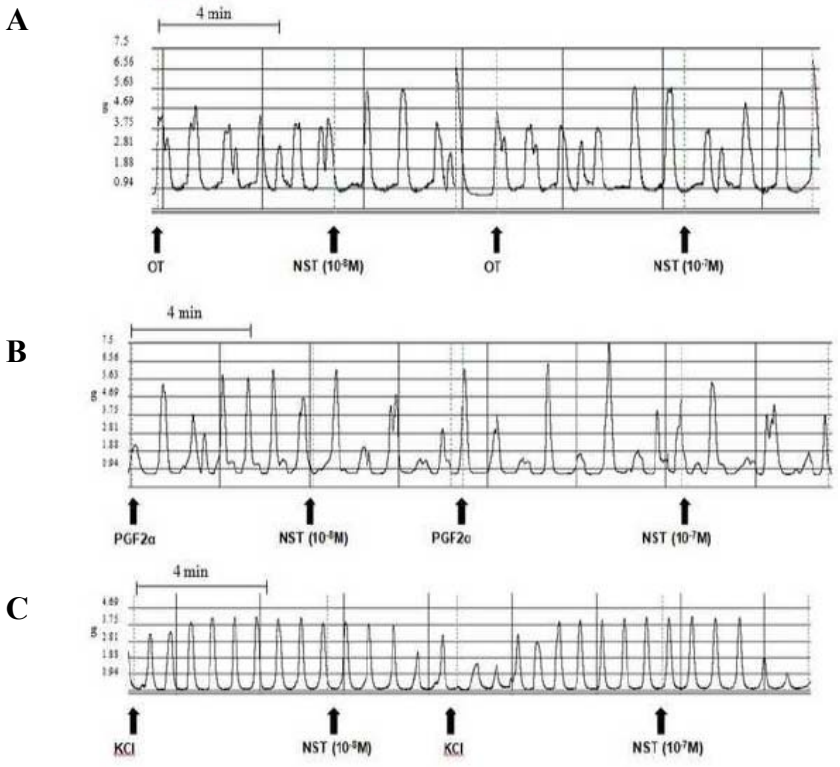

Fig. 1. Representative online recordings of the inhibitory effects of nocistatin (NST) and nociceptin (PNOC) on pregnant uterine contractions in vitro.
The contractions were elicited with 1 μM oxytocin (A) or with 1 μM prostaglandin F2 alpha (B) or with 25 mM KCl (C).

N/OFQ was able to potentiate the relaxant effect of NST, which was an interesting finding since NST counteracts the effects of N/OFQ relating to their actions in the central nervous system [Okuda-Ashitaka and Ito, 2000; Okuda-Ashitaka et al. 1998].

Table 2. EC_{50} and maximum 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).

Substance	EC_{50} ($\times 10^{-10}$ M ± S.E.M.)		E_{max} (% ± S.E.M.)	
NST (on $PGF_{2\alpha}$-evoked contraction)	10.05 ± 1.12		25.97 ± 3.15	
NST + N/OFQ	0.59 ± 0.13	*	33.98 ± 4.19	*
NST (on KCl-evoked contraction)	194.1 ± 117.3		56.10 ± 4.82	
NST + N/OFQ	428.0 ± 250.8	ns	65.78 ± 9.42	*
NST + N/OFQ + NX	58.2 ± 36.3	**b**	40.33 ± 2.73	**a**

*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.

Since the receptor target of NST is still unknown, we examined the involvement of opioid receptors in the peripheral actions of NST. Naloxone (NX) decreased the contraction inhibiting effect of NST (**Table 3**), which was a surprise, opposing previous studies [Johnson and Connor, 2007; Fantin et al., 2007] that excluded the direct binding of NST to opioid receptors. Since it was demonstrated by others that NX induces inward Ca^{2+} currents [Kai et al., 2002], we wondered whether the inhibitory effect of NX on NST-induced uterus relaxation is mediated by the opening of inward rectifying Ca^{2+} channels. In Ca^{2+}-poor environment, NX did not decrease the uterus-relaxant effect of NST (**Table 3**), suggesting that without an inward Ca^{2+} current, NX could not overcome the relaxing effect of NST.

Hereby, we provided evidence that the site of interaction between NST and NX is rather the inward rectifying Ca^{2+} channels.

Table 3. EC_{50} and maximum 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 Ca^{2+}-poor de Jongh solutions (n=6).

Substance	EC_{50} ($\times 10^{-10}$ M ± S.E.M.)		E_{max} (% ± S.E.M.)	
NST (1 mM Ca^{2+})	194.1 ± 117.3	**a**	56.10 ± 4.82	
NST + NX (1 mM Ca^{2+})	106.2 ± 48.8	ns; **a**	24.78 ± 3.08	***
NST (0.5 mM Ca^{2+})	0.3 ± 0.1		47.21 ± 4.27	
NST + NX (0.5 mM Ca^{2+})	0.5 ± 0.2	ns	47.82 ± 4.85	ns

***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. **a**: p<0.05; significances are expressed relative to the same substance in a different Ca^{2+}-containing de Jongh solution.

Additional experiments were done in the presence of the $K_{Ca}1.1$ channel (Ca^{2+}-dependent K^+ channel)-selective blocker paxilline (PAX), against spontaneous uterine contractions in standard de Jongh buffer, in order to investigate the participation of the outward rectifying K^+ channels in mediating the effects of NST. In the uterus, the plentiful $K_{Ca}1.1$ channels play an important role in decreasing depolarization, thereby relaxing the uterine smooth muscle. PAX inhibited the uterus-relaxant effect of NST (E_{max} decreased from 33% ± 3% to 11% ± 4%) as evidence that the Ca^{2+}-dependent K^+ channels play a role in the intracellular signalling of NST.

The possible involvement of CGRP, a sensory neuropeptide, in the actions of NST was also tested on uterine tissue. The rationale for these experiments was that inflammatory mediators are known to play a role in the initiation of labour, yet some of them (e.g. calcitonin gene-related peptide; CGRP) exhibit utero-relaxant activity among their various effects. Besides, pioid-like nociceptive peptides have been reported to release neurotransmitters, among them CGRP, from capsaicin-sensitive primary sensory neurons [Peiser et al., 2000; Helyes et al., 1997]. Although the uterus undergoes a gestation induced denervation, the process mainly affects adrenergic and not sensory units, leaving CGRP-containing nerves mainly intact [Klukovits A et al., 2004]. We investigated the effect of NST either on capsaicin-induced CGRP-depleted uterus samples or on CGRP-reloaded uterus samples (**Table 4**).

Table 4. EC_{50} and maximum 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).

Substance	EC_{50} ($\times 10^{-11}$ M ± S.E.M.)		E_{max} (% ± S.E.M.)	
NST control	73.7 ± 47.3		48.72 ± 7.18	
NST preincubated with capsaicin	not converged	ns	6.14 ± 4.79	**
NST preincubated with capsaicin and CGRP	160.6 ± 57.3	ns	35.05 ± 7.38	**

**p<0.01, ns: nonsignificant. 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.

Neuropeptide depletion from the capsaicin-sensitive primary afferents was induced with capsaicin, after which the maximum uterus-relaxant effect of NST was decreased. When the neuropeptide depletion was followed by the addition of CGRP, the maximum uterus-relaxant effect of NST was higher than after incubation with capsaicin. Therefore, the re-addition of CGRP restored the relaxant effect of NST as compared with the control. We assume that CGRP is an important factor in the uterus-relaxant effect of NST. NST, similarly to opioid peptides and N/OFQ, may promote the release of neuropeptides from sensory nerves [Moran et al., 2000].

2.4. Measurement of uterine cAMP accumulation by enzyme immunoassay (EIA)

For further investigation of the mechanism by which NST inhibits uterine contractions, the effect of NST on uterine cAMP accumulation was measured by EIA. We detected moderate elevation of cAMP levels in the presence of NST, similarly as in the case of N/OFQ. 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]. In correlation with the 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.

3. Experiments on isolated human uterus specimens

3.1. Human subjects

Biopsy specimens of human myometrial tissue were obtained from the Department of Obstetrics and Gynecology, University of Szeged, with the help of Zsolt Kormányos, MD, PhD, as it was indicated in the proposal. Uterine strip excisions were made at cesarean

sections, with the approval of The Ethical Committee of Albert Szent-Györgyi Clinical Center (registration number: 114/2009); and with the uninfluenced informed consent from the pregnant women. Uterine specimens were grouped as (1) at full term pregnancy (37-41 weeks of gestation) and (2) at preterm birth (33-36 weeks). We note here, that preterm deliveries which end up with caesarean sections – and not spontaneous labour - were quite rare at our University Clinic. During the first (and only) year of the project, we could perform the RT-PCR studies and the basic in vitro contractility studies.

3.2. RT-PCR studies

The myometrial PNOC mRNA levels were significantly higher in preterm uterine samples as compared with samples from full term pregnancy.

3.3. In vitro contractility studies

In the isolated uterine rings, rhythmic contractions were elicited with 10^{-8} M oxy. 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. In the full-term human myometrium N/OFQ alone decreased uterine contractility concentration-dependently, which was further increased by NST. NST alone also showed uterus relaxant effect, which was not different in the presence of N/OFQ (**Table 5**). In the uterus specimens from preterm birth N/OFQ alone decreased the oxy induced uterine contractions, which effect was further increased by NST, providing the most pronounced inhibitory effect on the human samples. NST alone also showed uterus relaxant effect, which was not altered by N/OFQ.

Table 5. Maximum 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 from term and preterm human pregnancy *in vitro*.

	$\mathbf{E_{max}}$ (% ± S.E.M.)			$\mathbf{E_{max}}$ (% ± 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

*** $p<0.001$; significances are expressed relative to N/OFQ. ns: non-significant; significances are expressed relative to NST.

As we have previously shown the CGRP-liberating effect of NST as well as the cAMP-accumulating effect of N/OFQ and NST in the pregnant rat uterus [Klukovits et al., 2010; Deák et al. unpublished], these results may also explain the uterus-relaxant effect of N/OFQ and NST and their intracellular signalling in the human uterus. We can also conclude from the human in vitro experiments that NST administration must precede the administration of N/OFQ, in the aim to enhance the common uterus-relaxant effect. NST stimulates CGRP liberation, which effect is weak compared to the more prominent potassium channel opening effect caused by N/OFQ. When N/OFQ is administered first, the CGRP elevation cannot exceed the effect of potassium channel opening caused by standard-dose of N/OFQ.

4. Conclusions and remarks

Investigation of the actions of the nociceptive peptides NST and N/OFQ has provided substantial new information relating to the mediation of uterine contractions at term, either physiological or pathological. By completing the tasks of the project, we contributed to the current knowledge on the peripheral actions of N/OFQ and NST, which has mostly covered their role in pain processing by far.

Finding the mRNA for *PNOC* in the rat and human uterus confirmed a local regulatory role for NST and N/OFQ in uterine functions. We also justified in the rat uterus that *PNOC* mRNA is translated to both N/OFQ and NST, the latter being more abundant at term. Unfortunately, due to the restricted time of the project, we were not able to show evidence for the existence of specific binding sites for NST in the uterus, which would have been a significant step towards the identification of NST receptors.

Upon the results of the in vitro contractility assays on the pregnant rat uterus, we provided evidence that NST alone and also in combination with N/OFQ exert a relaxant effect. This mechanism was mediated in part by $K_{Ca}1.1$ channels (Ca^{2+}-dependent K^+ channels) and consequent hyperpolarization, and by the release of the sensory neuropeptide CGRP. The increased cAMP-accumulation by NST probably plays a minor role in its contraction inhibiting effect. We also proved the efficacy of N/OFQ and NST on human specimens (at term and preterm labour) to decrease uterine contractions in vitro. If N/OFQ and NST are co-administered, NST should be added first in order to maximize the relaxant effect.

Further experiments on human tissue are necessary to allow conclusions on the relevance of the present findings as concerns human disease. Understanding this complex signaling

pathway, however, may provide an opportunity for the development of novel treatments for the inhibition of uterine contractions arising before the term of pregnancy.

The results of the project PD-100868 are going to be published in the near future, in 2 manuscripts:

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. *Under major revision at European Journal of Pharmacology; manuscript number: EJP-37241*

Deák BH, Klukovits A, Tekes K, Ducza E, Kormányos Z, Gáspár R. Uterus-relaxing effect of nociceptin and nocistatin: studies on preterm and term-pregnant human myometrium *in vitro. Manuscript written, to be published in Reproductive System and Sexual Disorders.*

On behalf of my co-workers and assistants, who helped me to accomplish this project, I am deeply grateful for the financial support provided by OTKA. I would also like to render thanks for their understanding and letting me finish the project earlier than it was supposed to.

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